18MBO43E

Core : Elective paper -IV Biotechnology

UNIT – 3

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Aim and scope rDNA technology and basic steps of Genetic engineering

Recombinant DNA technology

Recombinant DNA (r-DNA) technology has made a revolutionary impact in the area of human healthcare by enabling mass production of safe, pure and effective r-DNA expression products.

•Recombinant DNA is specifically from two or more DNA incorporated into a single molecule.

• Genetic engineering, recombinant DNA technology, genetic modification and gene splicing are terms are applied to the direct manipulation of an organisms gene.

• The development of these new technologies have resulted into production of large amount of biochemically products

r-DNA involves using microorganisms

1. **To create new pharmaceuticals**
2. **To create safer/ more effective version therapeutic agents**

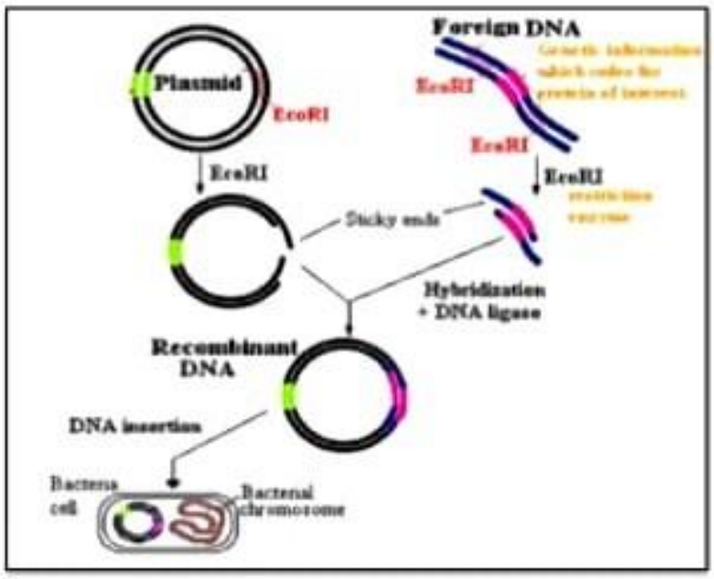
RECOMBINANT DNA (r DNA)

DNA that has been created artificially (not natural). DNA from two or more sources is incorporated into a single recombinant molecule

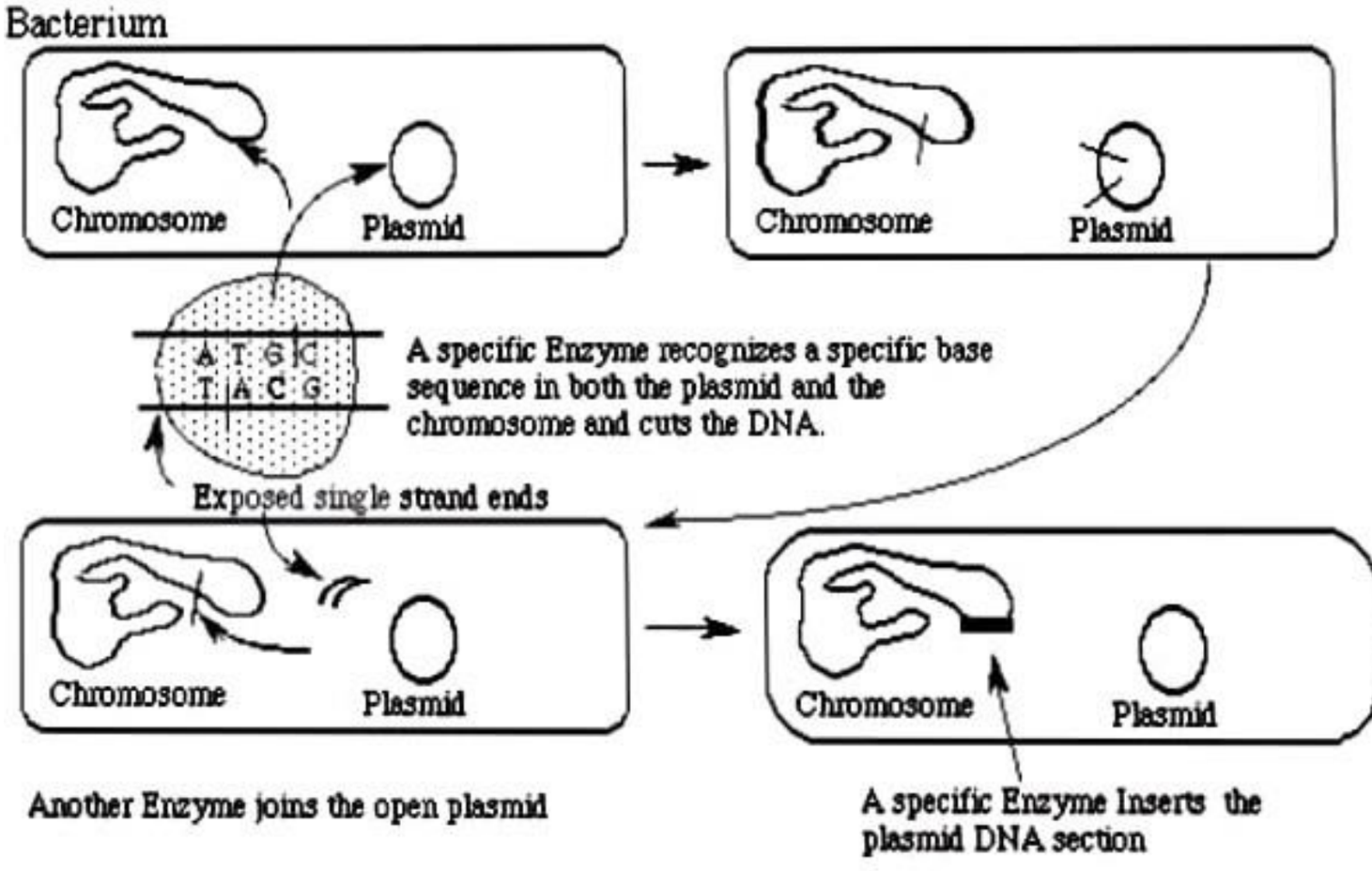
Recombinant DNA(rDNA) is a form of artificial DNA that is created by combining two or more sequences. It is made possible by two important enzymes. **Restriction enzymes** and **DNA Ligase** are the two principal tools, first used by Paul Berg in 1972,employed to alter DNA.

❑ **METHODS BY WHICH RECOMBINANT DNA IS MADE ARE:**

- TRANSFORMATION
- TRANSDUCTION
- CONJUGATION



Basic principle of recombinant DNA technology



Recombinant DNA technology

- A series of procedures used to recombine DNA segments.
- Under certain conditions, a recombinant DNA molecule can enter a cell and replicate.
- Recombinant DNA technology is one of the recent advances in biotechnology, which was developed by two scientists named Boyer and Cohen in 1973.
- The DNA is inserted into another DNA molecule called 'vector'.
- The recombinant vector is then introduced into a host cell where it replicates itself, the gene is then produced

In vitro recombination
Genetic engineering
Genetic surgery

HISTORY

- First breakthrough in **1960s.**
- In **1969, Herbert Boyer** isolated restriction enzyme **EcoRI** from E. coli.That cleaves the DNA between G and A in the base sequence **GAATTC.**

HISTORY

- **In 1970,**

  Howard Temin and **Davin Baltimore** independently discovered the enzyme reverse transcriptase from retroviruses.

  - This enzyme was used to construct a DNA called **complementary DNA (cDNA)** from any mRNA.

HISTORY

- In, **1972**

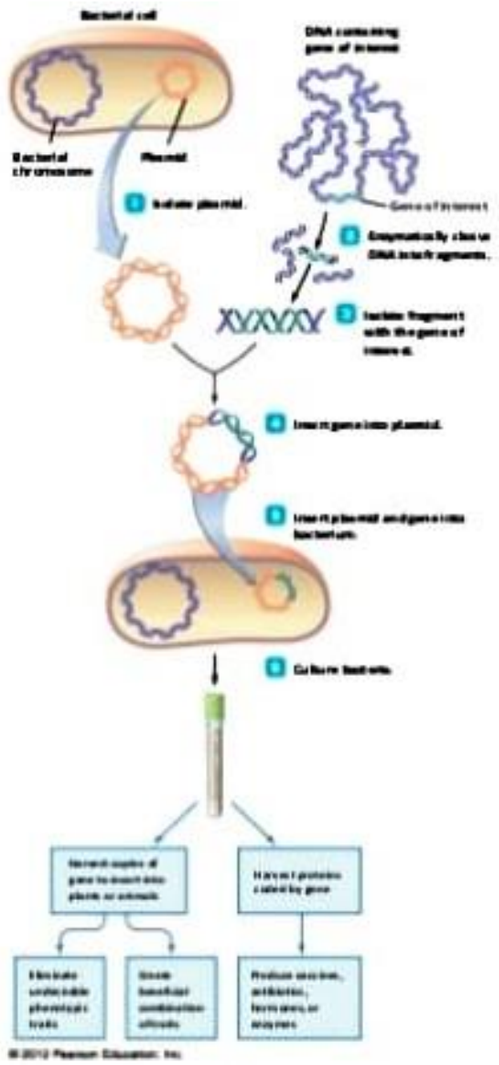
David Jackson, **Robert Symons** and **Paul Berg** successfully generated **rDNA molecules.**

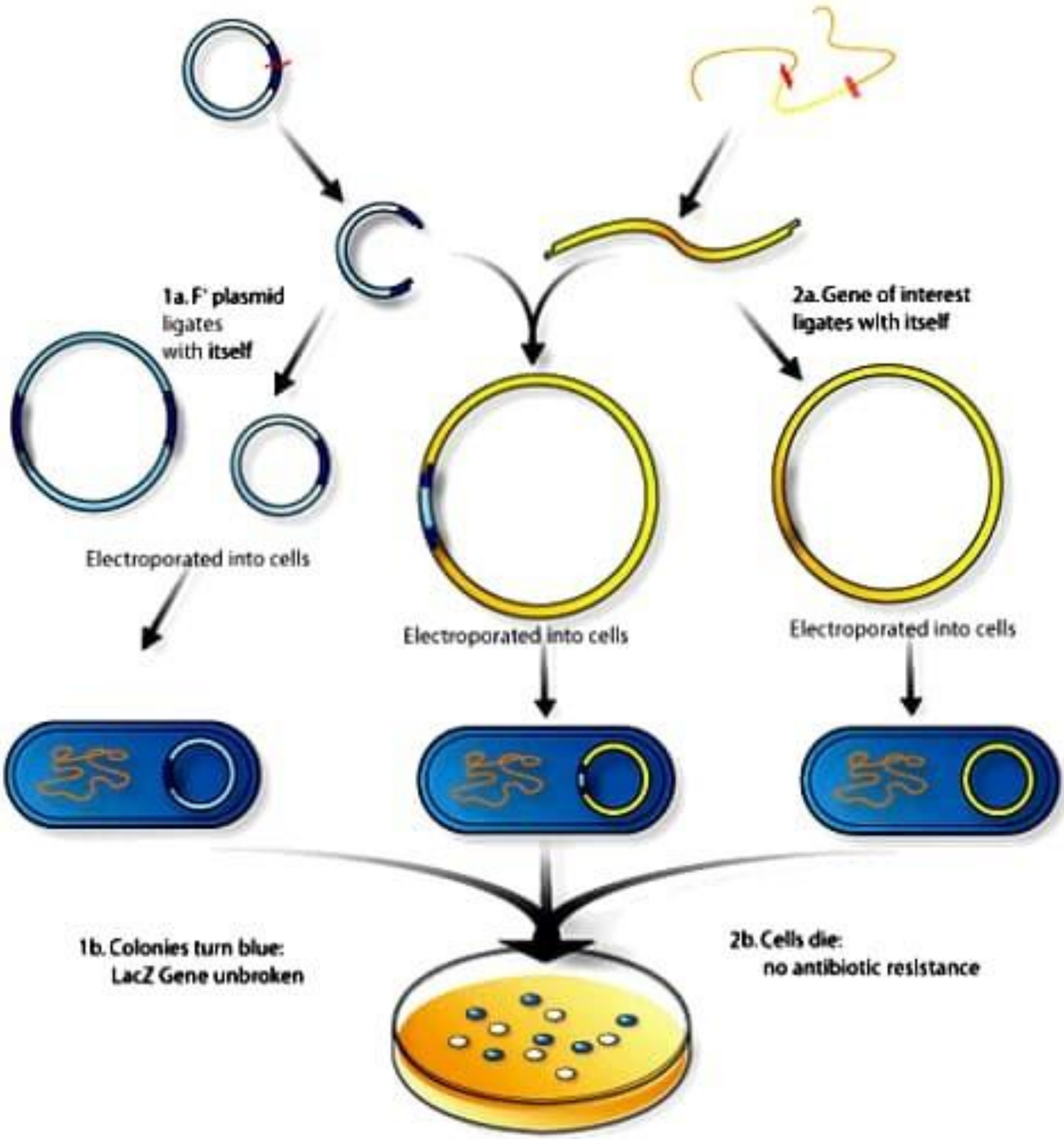
➢ They allowed the sticky ends of complementary DNA by using an enzyme **DNA ligase.**

The Role of Recombinant DNA Technology in Biotechnology

- **Recombinant DNA Technology**
  - Intentional modification of organisms' genomes for practical purposes
  - Three goals
    - Eliminate undesirable phenotypic traits
    - Combine beneficial traits of two or more organisms
    - Create organisms that synthesize products humans need

Overview of recombinant DNA technology




1a. F' plasmid
ligates
with itself
2a. Gene of interest
ligates with itself
Electroporated into cells
Electroporated into cells
Electroporated into cells
1b. Colonies turn blue:
LacZ Gene unbroken
2b. Cells die:
no antibiotic resistance

Applications of recombinant DNA technology

1. Insulin for diabetics.
2. Factor VIII for males suffering from haemophilia A.
3. Factor IX for haemophilia B.
4. Human growth hormone (HGH).
5. Erythropoietin (EPO) for treating anemia.
6. Several types of interferon.
7. Granulocyte –macrophage colony-stimulating factor(GSM-CSF) for stimulating the bone marrow after a bone marrow transplant.
8. Many monoclonal antibodies
9. Pharmaceutical and therapeutic applications
10. Gene therapy
11. Medical diagnosis
12. Xenotransplants

Recombinant r-DNA technology

Isolation of the gene of interest

Preparation of vector DNA and DNA to be cloned

Insertion of the gene to the vector molecule and ligation

Introduction of the vector DNA to the appropriate host cell

Amplification of the recombinant DNA molecule in the host cell

Advantages of Recombinant Technology

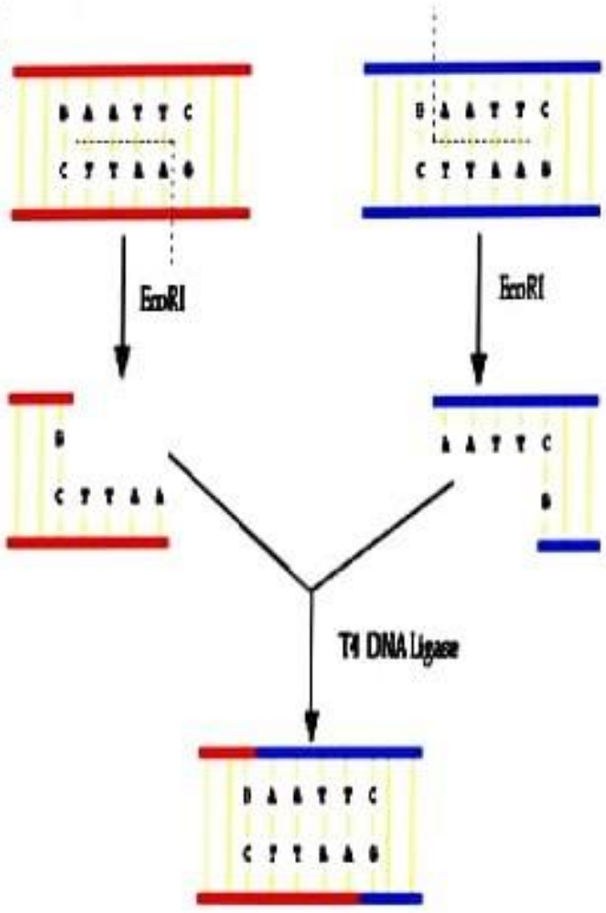
- Provide substantial quantity
- No need for natural or organic factors
- Unlimited utilizations
- Cheap
- Resistant to natural inhibitors

Disadvantages of recombinant technology

- Commercialized and became big source of income for businessman.
- Effects natural immune system of the body.
- Can destroy natural ecosystem that relies on organic cycle.
- Prone to cause mutation that could have harmful effects.
- Major international concern : manufacturing of biological weapons such as botulism & anthrax to target humans with specific genotype.
- Concerns of creating super – human race

Gentic Engineering

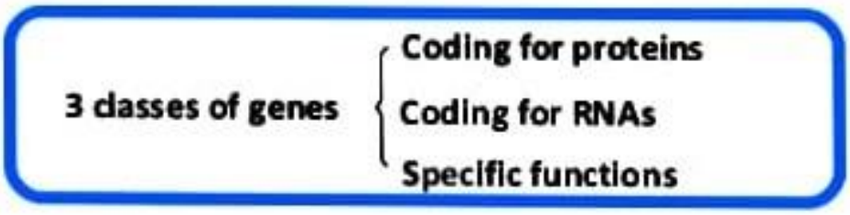
- **GE:** The technology entailing all processes of altering the genetic material of a cell to make it capable of performing the desired functions, such as producing novel substances.
- In other words: Genetic engineering is the deliberate, controlled manipulation of genes in an organism in order to upgrade that organism.
- In genetic engineering, recombination can also refer to artificial and deliberate recombination of pieces of DNA, from different organisms, creating what is called recombinant DNA.



Gene

Gene: A gene is a basic unit of heredity in a living organism.

It is "*a locatable region of genomic sequence, corresponding to a unit of inheritance, which is associated with regulatory regions, transcribed regions, and or other functional sequence regions* ". [1]



Genes hold the information to build and maintain an organism's cells and pass genetic traits to offspring. [2]

- **Allele: Each gene can have different alleles.**

- **An allele (from the Greek allelos, meaning each other) is *one of two or more forms of the DNA sequence of a particular gene.***
  E.g. Diploid , Triploid etc.

- **The vast majority of living organisms encode their genes in long strands of DNA.**

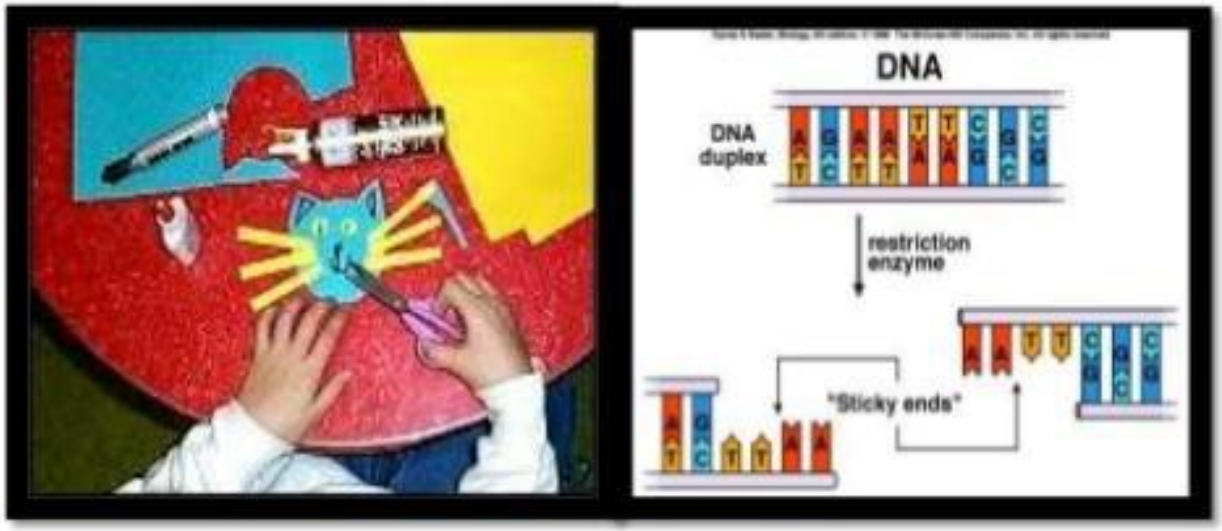
- **The most common form of DNA in a cell is in a double helix structure**

- **RNA is common as genetic storage material in viruses, in mammals in particular RNA inheritance has been observed very rarely.**

THANK YOU

Enzymes used in Genetic Engineering

- Cutting and pasting are two of the first skills children learn, and the tools they use are scissors and glue.



- Similarly, **cutting DNA** and **pasting DNA** fragments together typically are among the first techniques learned in the molecular biology lab and are **fundamental to all recombinant DNA work**.

Such manipulations of DNA are conducted by a toolkit of enzymes:

- restriction endonucleases are used as molecular scissors,
- DNA ligase functions to bond pieces of DNA together, and
- a variety of additional enzymes that modify DNA are used to facilitate the process.

DNA modifying enzymes

- Restriction enzymes and DNA ligases represent the **cutting and joining functions** in DNA manipulation.

- All other enzymes involved in genetic engineering fall under the broad category of enzymes known as **DNA modifying enzymes**.

- These enzymes are involved in the degradation, synthesis and alteration of the nucleic acids.

WHAT IS AN ENZYME?

- Enzymes are proteins and certain class of RNA (ribozymes) which enhance the rate of a thermodynamically feasible reaction and are not permanently altered in the process.

Molecular Scissors

Restriction enzymes are molecular scissors

RESTRICTION ENZYMES

- A restriction enzyme (or restriction endonuclease) is an enzyme that cuts double-stranded or single stranded DNA at specific recognition nucleotide sequences known as restriction sites.

Property of restriction enzymes

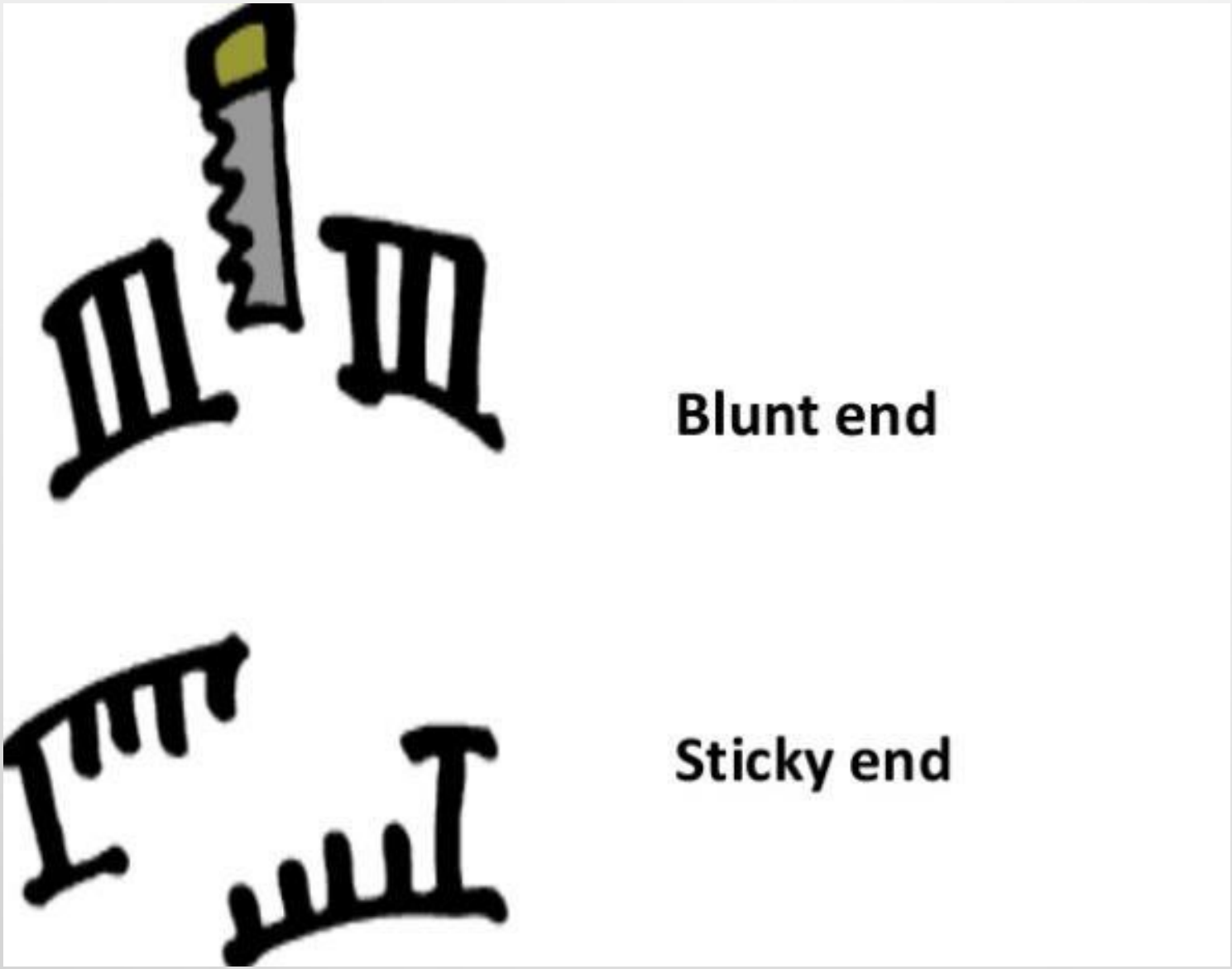
- They break the phosphodiester bonds that link adjacent nucleotides in DNA molecules.

HOW RESTRICTION ENZYMES WORKS?

- Restriction enzymes **recognize a specific sequence of nucleotides**, and produce a double-stranded cut in the DNA, these cuts are of two types:

- **BLUNT ENDS**.

- **STICKY ENDS.**


Blunt end
Sticky end

BLUNT ENDS

- These blunt ended fragments can be joined to any other DNA fragment with blunt ends.

- Enzymes useful for certain types of DNA cloning experiments

"STICKY ENDS" ARE USEFUL

DNA fragments with complimentary sticky ends can be combined to create new molecules which allows the **creation and manipulation of DNA sequences from different sources.**

- While recognition sequences vary widely , with lengths between 4 and 8 nucleotides, many of them are palindromic.

PALINDROMES IN DNA SEQUENCES

Genetic palindromes are similar to verbal palindromes. A palindromic sequence in **DNA is one in which the 5' to 3' base pair sequence is identical** on both strands (the 5' and 3' ends refers to the chemical structure of the DNA).

PALINDROME SEQUENCES

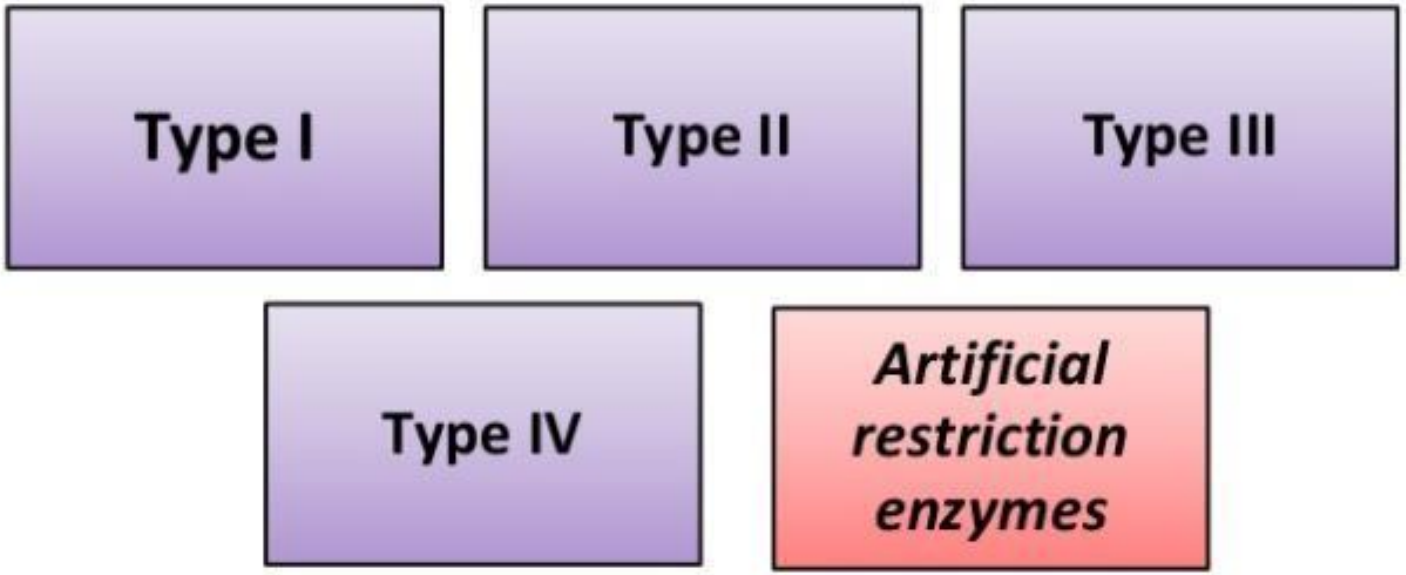
- The mirror like palindrome in which the same forward and backwards are on a single strand of DNA strand, as in GTAATG
- The Inverted repeat palindromes is also a sequence that reads the same forward and backwards, but the forward and backward sequences are found in complementary DNA strands (GTATAC being complementary to CATATG)
- **Inverted repeat palindromes are more common** and have greater biological importance than mirror-like palindromes.

TYPES OF RESTRICTION ENZYMES

- Restriction endonucleases are categorized into three general groups.

- **Type I**
- **Type II**
- **Type III**

TYPES OF RESTRICTION ENZYMES



These types are categorization based on:

- Their composition.
- Enzyme co-factor requirement.
- The nature of their target sequence.
- Position of their DNA cleavage site relative to the target sequence.

Type 1

- Capable of both restriction and modification activities
- The co factors S-Adenosyl Methionine(AdoMet), ATP, and mg++are required for their full activity
- Contain:
  - two R(restriction) subunits
  - two M(methylation) subunits
  - one S(specifity) subunits
- Cleave DNA at random length from recognition sites

Type II

- These are the most commonly available and used restriction enzymes
- They are composed of only one subunit.
- Their recognition sites are usually undivided and palindromic and 4-8 nucleotides in length,
- They recognize and cleave DNA at the same site.
- They do not use ATP for their activity
- They usually require only mg2+ as a cofactor.

Type III

- Type III restriction enzymes cut DNA about 20-30 base pairs after the recognition site.
- These enzymes contain more than one subunit.
- And require AdoMet and ATP cofactors for their roles in DNA methylation and restriction

Type IV

- Cleave only normal and modified DNA (methylated, hydroxymethylated and glucosyl-hydroxymethylated bases).
- Recognition sequences have not been well defined
- Cleavage takes place ~30 bp away from one of the sites

ARTIFICIAL RESTRICTION ENZYMES

- Generated by fusing a natural or engineered DNA binding domain to a nuclease domain
- can target large DNA sites (up to 36 bp)
- can be engineered to bind to desired DNA sequences

Plasmid vector

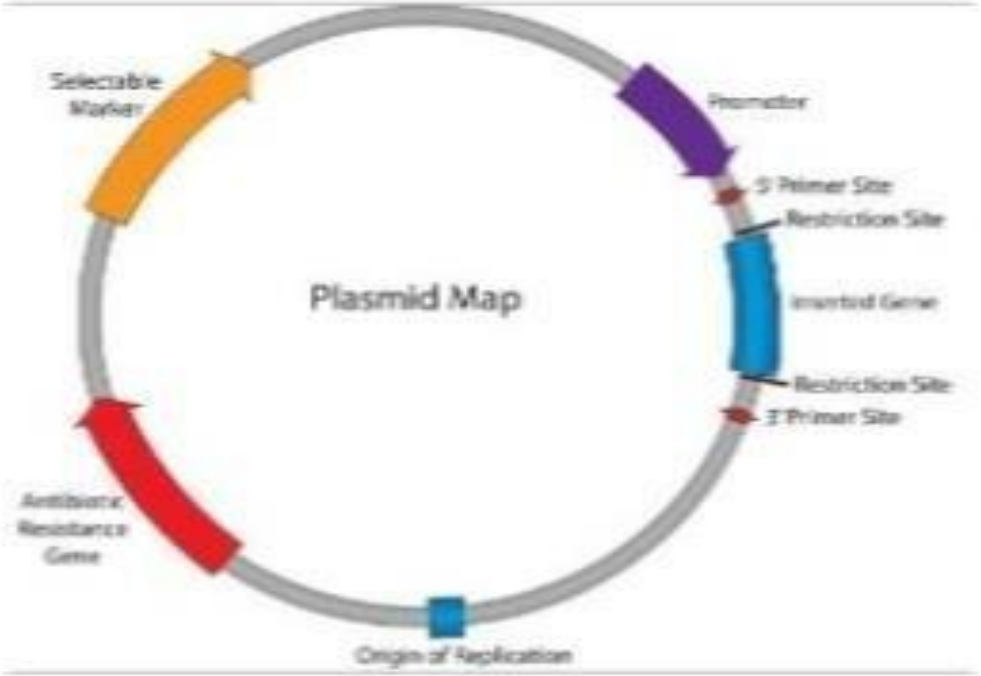
CONTENTS

INTRODUCTION

- A plasmid is a small, circular, extrachromosomal double stranded DNA that has the capacity to replicate independently.
- Discovered by Laderberg in 1952.
- It naturally occur in bacteria, however sometimes present in archaea and eukaryotes.
- The genes carried in plasmid benefit the survival of the organism by providing them with genetic advantages like antibiotic resistance etc. under certain situation or particular conditions.
- They provide mechanism for horizontal gene transfer within a population of microbes and thus provide a selective advantage under a given environmental state.

ELEMENTS OF PLASMIDS

- Origin of replication: it is the DNA sequence which directs initiation of plasmid replication by recruiting bacterial transcriptional machinery.
- Antibiotic resistance gene: these genes allows for selection of plasmid containing bacteria by providing a survival advantage to the bacterial host.
- Multiple cloning site: this is the short segment containing several restriction enzyme sites, enabling easy insertion of foreign DNA.



PLASMID CONFORMATIONS

1. Nicked open circular DNA
2. Relaxed circular DNA
3. Linear DNA
4. Supercoiled or covalently closed circular DNA
5. Supercoiled denatured DNA

PROPERTIES

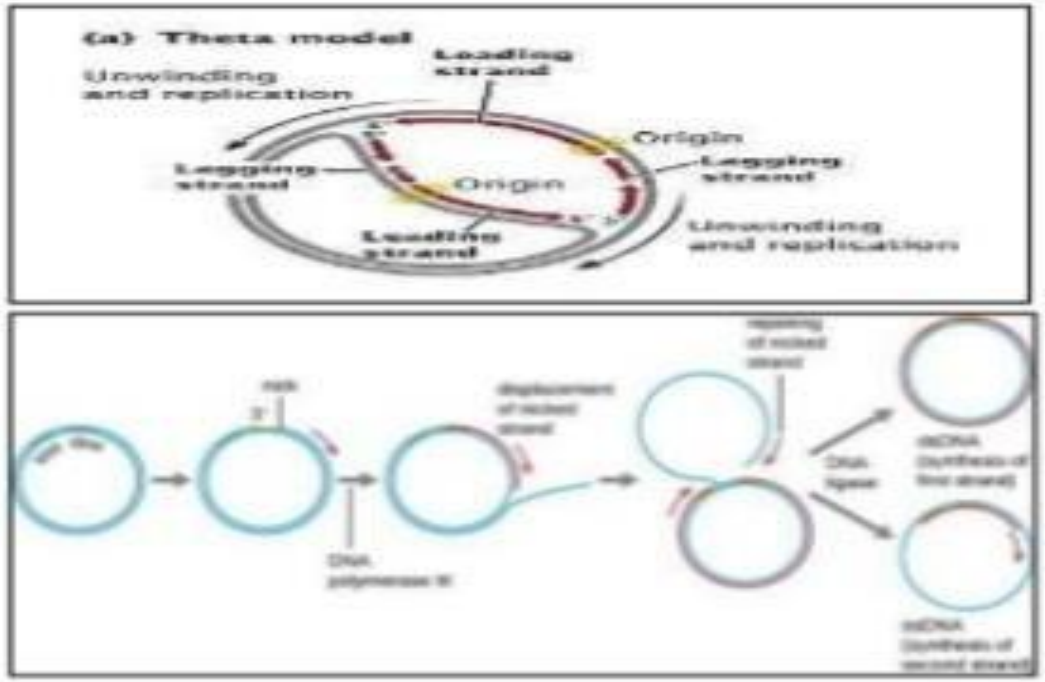
- Specific to one or a few particular bacteria.
- Replicate independently and code for their own transfer.
- Do not cause damage to cells and generally are beneficial, do not have extracellular forms and exist inside cells simply as free and typically circular DNA.
- Size ranges from 1 kbp to several mbp.
- Number of plasmids in an individual cell may vary, ranging from one to several hundreds, denoted by copy number.
- Genes carried by plasmid encodes traits for antibiotic resistance or resistance to heavy metal.
- Some produces virulence factor that help in defence or nutrient utilization.
- Plasmids can also provide bacteria with the ability to fix nitrogen.
- Some also exhibits properties like sulphur utilization, hydrocarbon degradation, drug resistance etc.

- Insert: it is the foreign DNA cloned into the multiple cloning site.
- Promoter region: it drives the transcription of the foreign insert.
- Selectable marker: it is used to select for cells that has successfully taken up the plasmid for the purpose of expressing the insert.
- Primer binding site: it is the site for binding of short single stranded DNA sequence, used as an initiation point for PCR amplification or sequencing of the plasmid.

PLASMID REPLICATION

Plasmids replicate autonomously because they have their own replication origins.

- Most plasmids in gram-negative bacteria replicate in a manner similar to the replication of bacterial chromosome involving initiation at the replication origin site and bidirectional replication around the DNA circle giving a theta (Θ) intermediate.
- Most plasmids of gram-positive bacteria replicate by a rolling circle mechanism.



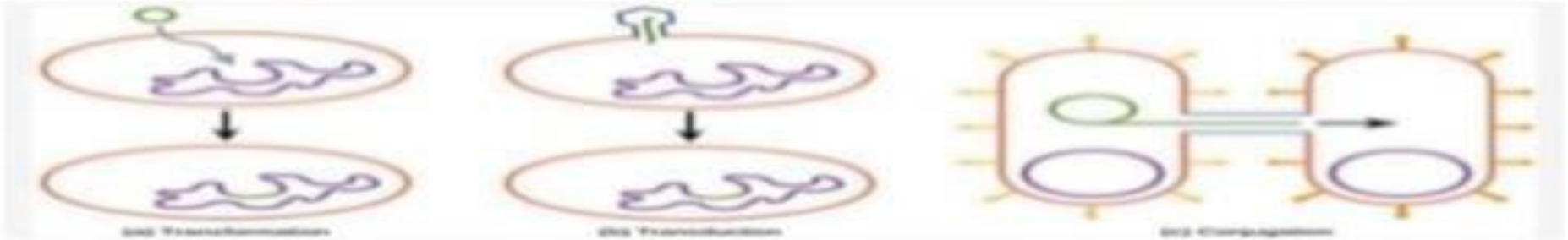
MODE OF PLASMID TRANSFER

The genetic information encoded in a plasmid of bacteria is transferred across a broad range of microorganism via-

1.Transformation: requires competent cells which are ready to accept extracellular plasmid and further stable replication inside host cell.

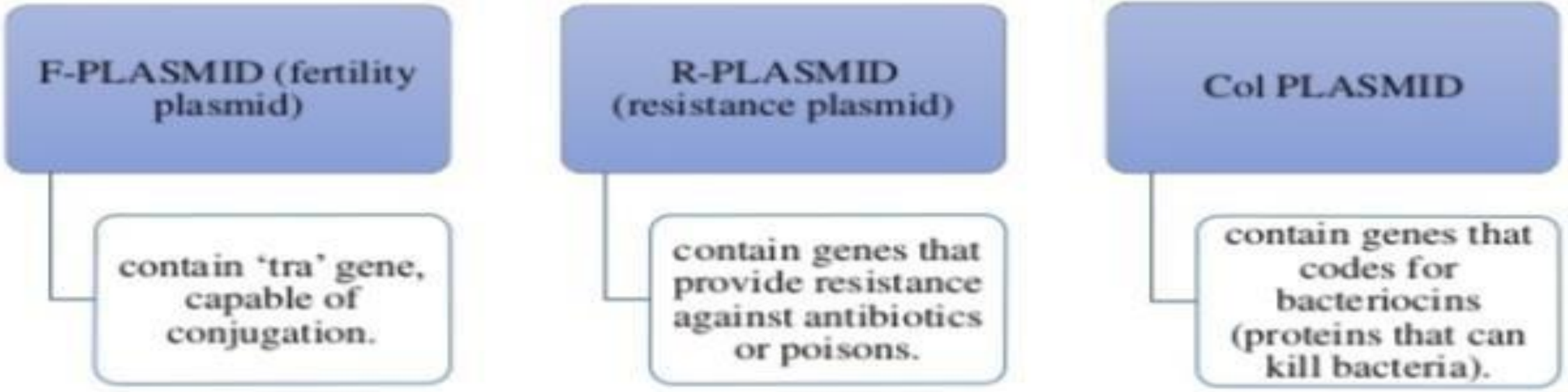
2.Transduction: plasmid mediated gene transfer through bacteriophages.(can be generalised or specialised)

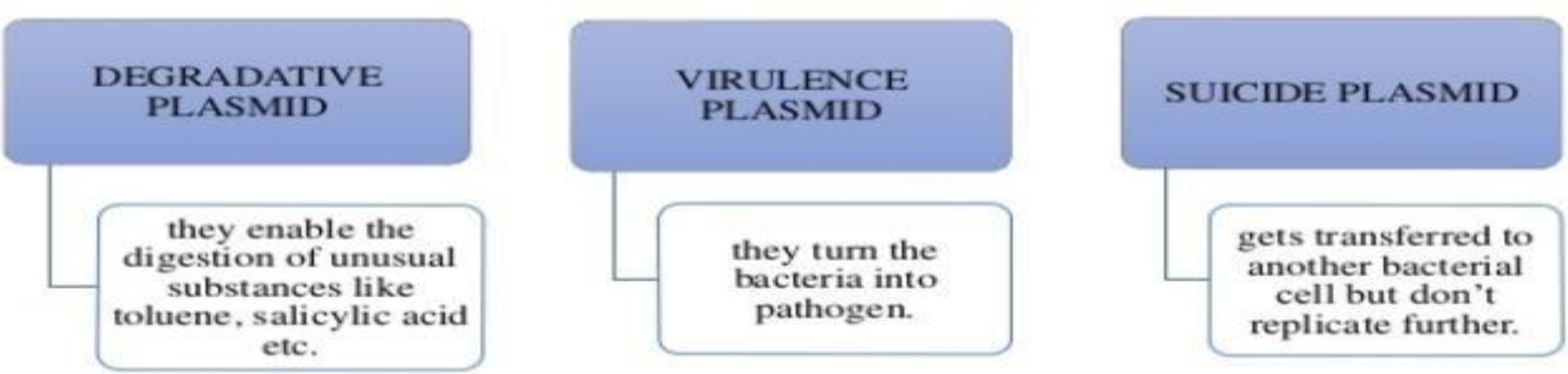
3.Conjugation: transfer through cell to cell contact of donor and recipient cell, requires DNA metabolism of donor cell.



CLASSIFICATION

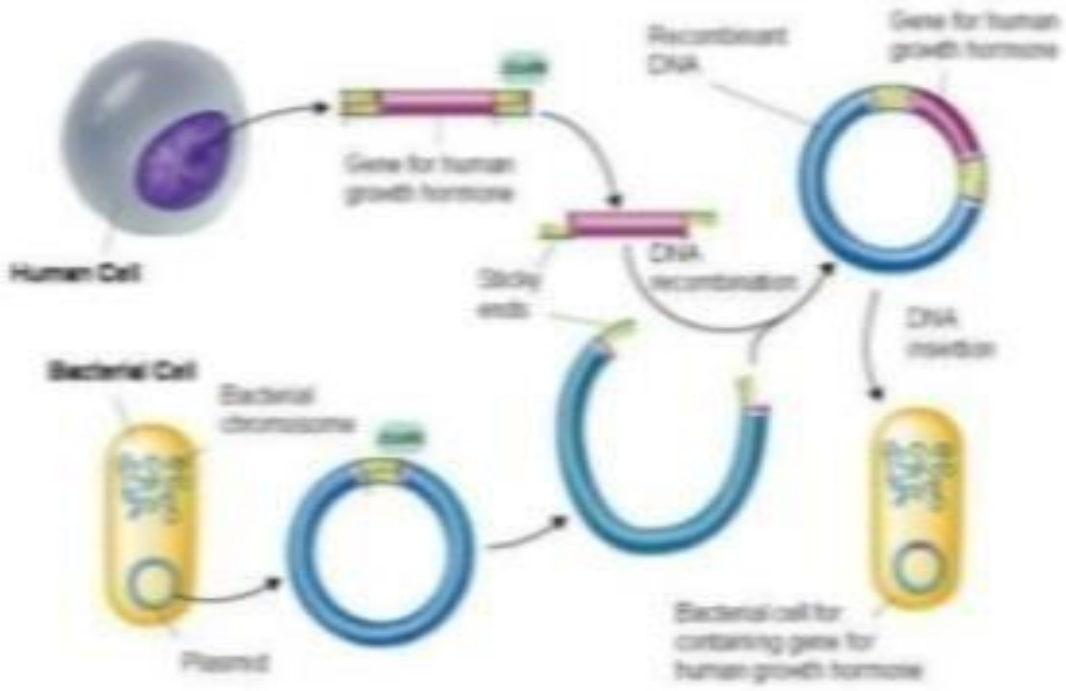
Based on their functions, plasmids are classified as follows-




DEGRADATIVE PLASMID
they enable the digestion of unusual substances like toluene, salicylic acid etc.
VIRULENCE PLASMID
they turn the bacteria into pathogen.
SUICIDE PLASMID
gets transferred to another bacterial cell but don't replicate further.

PLASMIDS AND RECOMBINANT DNA TECHNOLOGY

- Recombinant DNA technology is joining together of DNA molecules from two different species that are inserted into a host organism to produce new genetic combinations that are of value to science, medicine, agriculture, and industry.
- Artificially constructed plasmids are used as vectors in genetic engineering to clone or amplify or express particular genes.
- When a single recombinant DNA molecule composed of vector and inserted DNA molecule is introduced into host cell, the inserted DNA is reproduced along with the vector, producing large number of recombinant DNA molecule that include the fragment of DNA molecule originally linked to the vector



USES

1. CLONING
2. PROTEIN PRODUCTION
3. GENE THERAPY
4. GENETIC DISEASE MODELS
5. PLASMID DNA VACCINES

IMPORTANCE OF PLASMID

- Easy to work with due to convenient size for physical isolation and manipulation, easy to create and modify plasmids containing the genetic element that one is interested in.
- Independent origin of replication allows plasmid replication in the cell to proceed independently from direct chromosomal control.
- Multiple copy number makes them to be present in the cell in several copies so that amplification of the plasmid DNA becomes easy
- Presence of selectable markers such as antibiotic resistance genes, which make detection and selection of plasmid-containing clones easier.

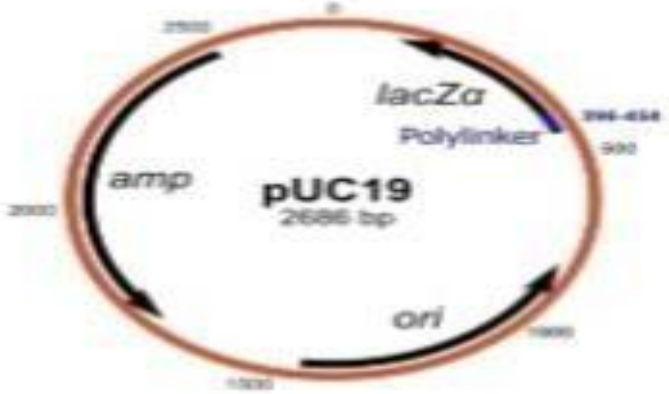
Continued...

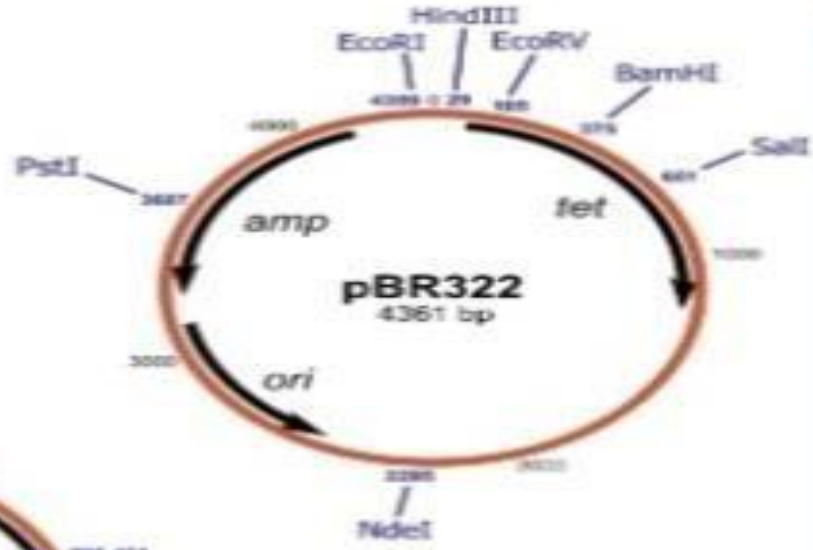
- Stable for long term either as purified DNA or within bacterial cell preserved as glycerol stocks.
- Functional in many species including plants, worms, mice and even cultured human cells and useful for a diverse set of applications like investigation of promoters, small RNAs or other genetic elements.

DRAWBACK: Less useful for cloning large segment of DNA (>10kbp).

EXAMPLES

- pBR322
- pBR327
- pBR325
- pBR328
- pUC8
- pUC9
- pUC12
- pUC13






THANK YOU..!!

Phage vectors

INTRODUCTION

- Bacteriophage are viruses that are capable of infecting bacteria.
- In situation, the phage chromosome integrates into the bacterial chromosome and multiplies with the latter prophage.
- The prophage may dissociate from the bacterial chromosome and follow the lytic cycle

- Several bacteriophages are used as cloning vectors, the most commonly used E.Coli phages being (lambda) and M13 phages.

Phage vector

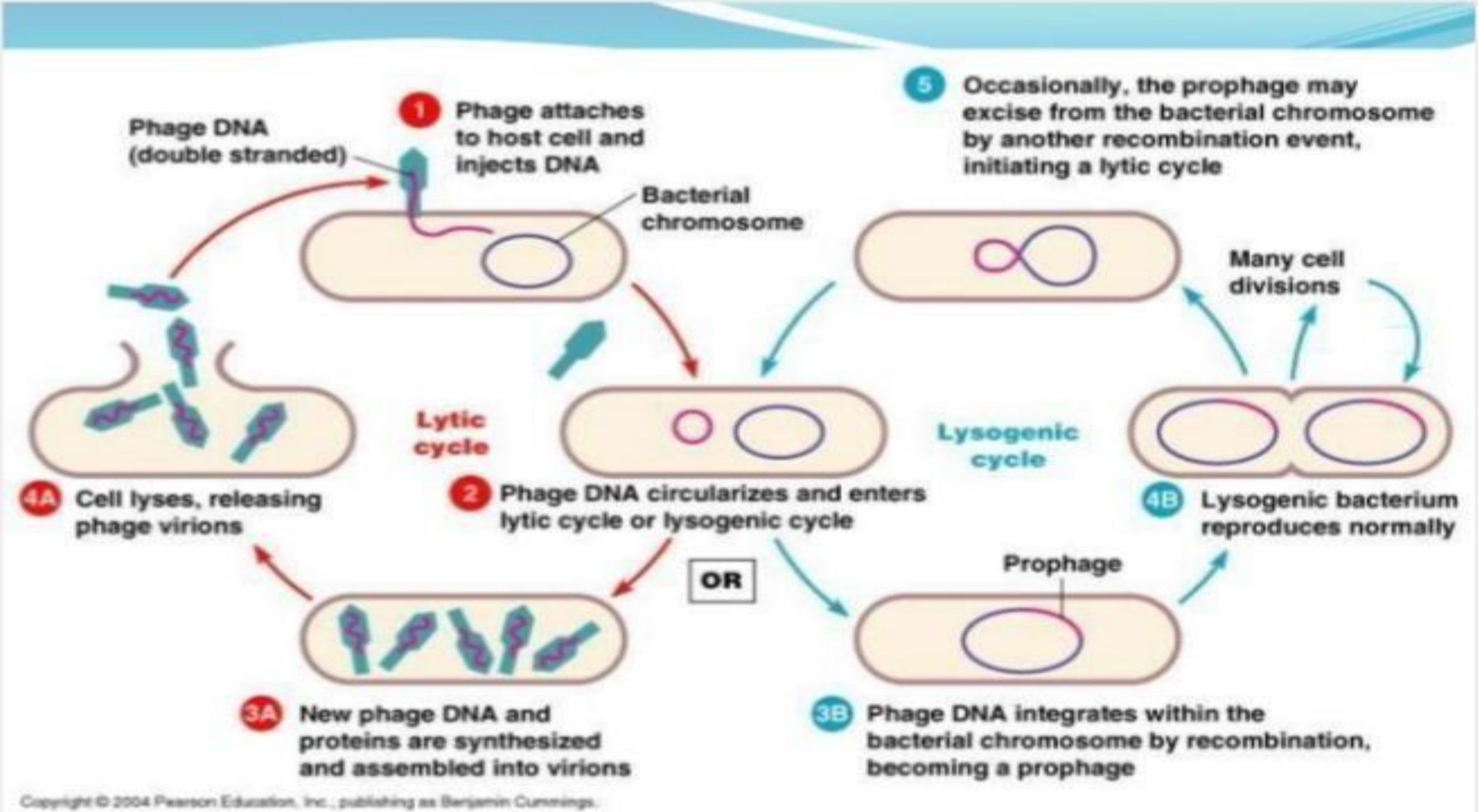
- It is a bacterial virus, or **bacteriophage, that infects the** bacterial species *Escherichia coli.*
- This virus is a **temperate phage**.
- Temperate phages are basically
- bacteriophages which can choose between a **lytic and lysogenic** pathway of development.

Lifecycle

Lysogenic cycle lytic cycle

- The viral DNA exists as a **separate molecule** within the bacterial cell.
- Replicates separately from the host bacterial DNA.

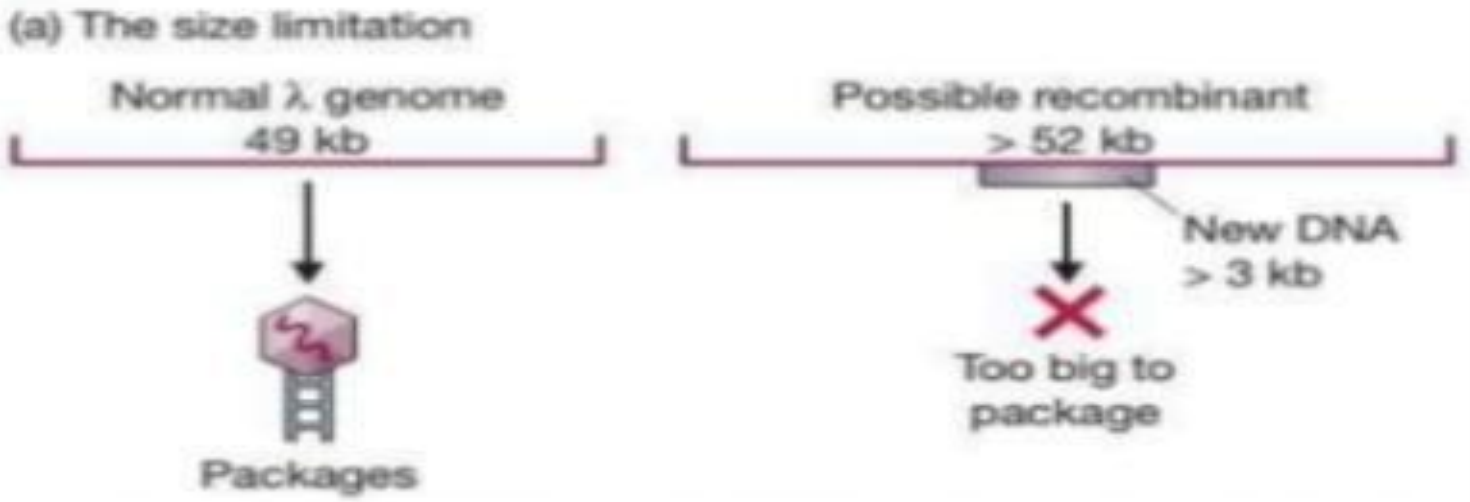
- Viral **DNA integrates** with bacterial DNA.
- Replicates with the host DNA

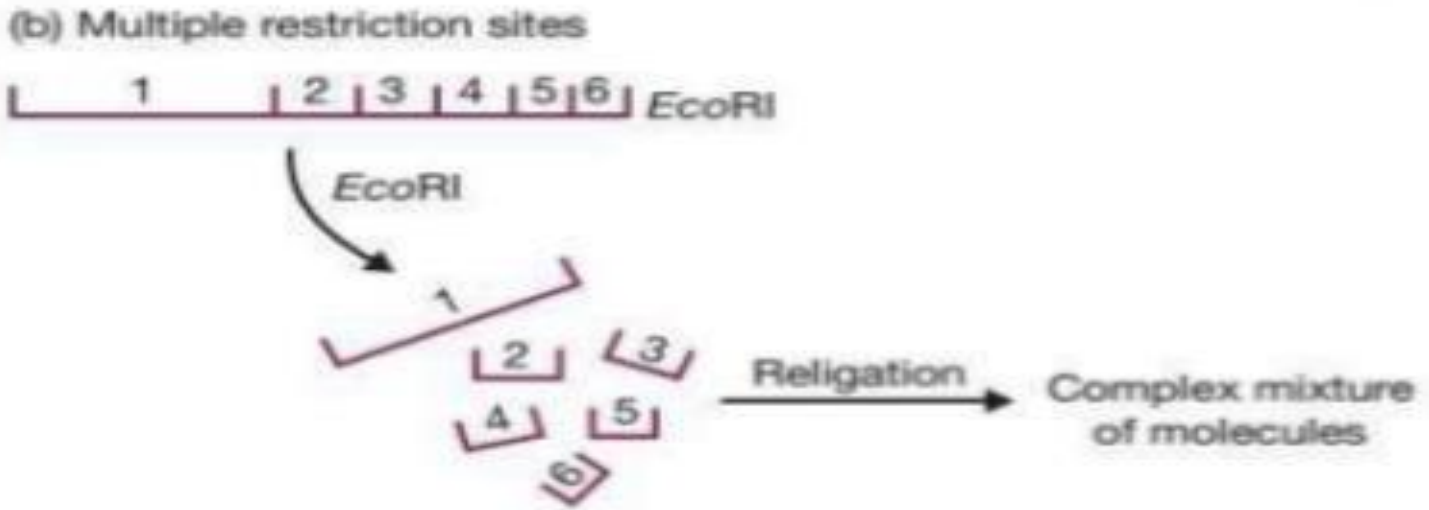


Cloning vector

- Two problems had to be solved before lambda based cloning vectors could be developed:
- (1)The DNA molecule can be increased in size by only about 5%, representing the addition of only 3 kb of new DNA.
- The total size of the molecule is more than52 kb, then it cannot be packaged into the head structure and infective phage particles are not formed.
- This severely limits the size of a DNA fragment that can be inserted into an unmodified vector

- (2)The lambda genome is so large that it has more than one recognition sequence for virtually every restriction endonuclease.
- Restriction cannot be used to cleave the normal lambda molecule in a way that will allow insertion of new DNA,
- Because the molecule would be cut into several small fragments that would be
- very unlikely to re-form a viable lambda genome on re ligation


(a) The size limitation
Normal λ genome
49 kb
Packages
Possible recombinant
> 52 kb
New DNA
> 3 kb
Too big to
package

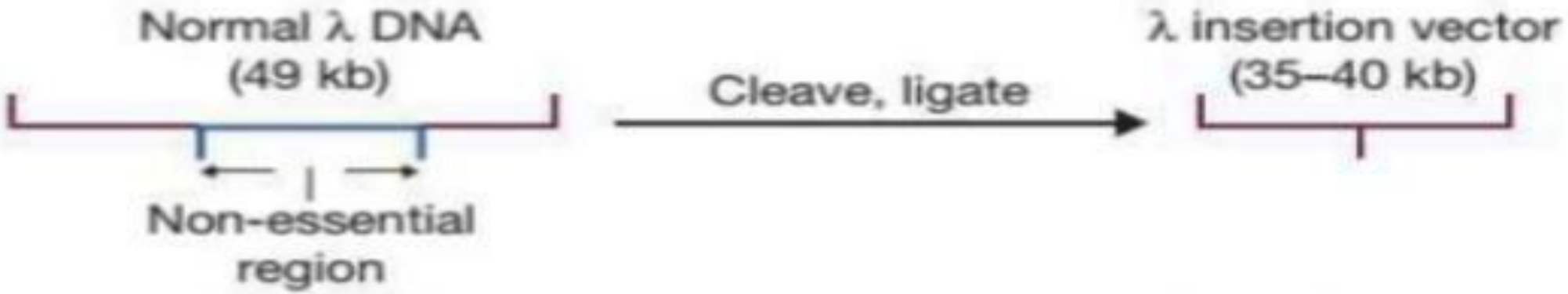

(b) Multiple restriction sites
1
2
3
4
5
6
EcoRI
EcoRI
Religation
Complex mixture
of molecules

Replacement vectors

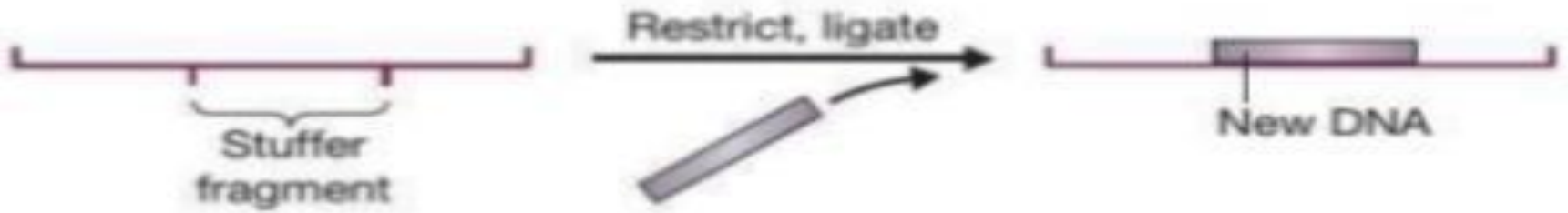
- A lambda replacement vector has two recognition sites for the restriction endonuclease used for cloning.
- These sites flank a segment of DNA that is replaced by the DNA to be cloned
- Often the replaceable fragment (or "stuffer fragment") carries additional restriction sites that can be used to cut it up into small pieces so that its own re-insertion during a cloning experiment is very unlikely.

- Replacement vectors are generally designed to carry larger pieces of DNA than insertion vectors can handle.
- Recombinant selection is often on the basis of size, with non-recombinant vectors being too small to be packaged into lambda phage heads

(a) Construction of a λ insertion vector



(a) Cloning with a λ replacement vector

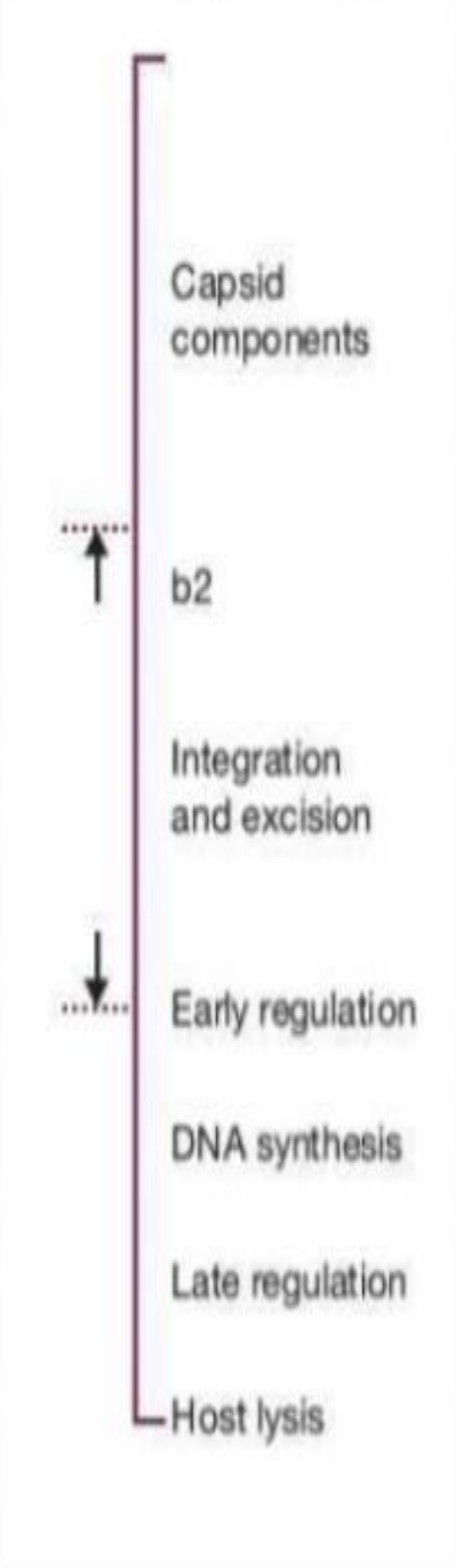


Insertion vector

- With an insertion vector a large segment of the non-essential region has been deleted, and the two arms ligated together.
- An insertion vector possesses at least one unique restriction site into which new DNA can be inserted.
- The size of the DNA fragment that an individual vector can carry depends, of course, on the extent to which the non-essential region has been deleted.
- Two popular insertion vectors are

Segments of the lambda genome can be deleted without impairing viability

- Large segment in the central region of the lambda DNA molecule can be removed without affecting the ability of the phage to infect E. coli cells.
- Removal of all or part of this non-essential region, between positions 20 and 35 on the map decreases the size of the resulting lambda molecule by up to 15 kb.
- This means that as much as 18 kb of new DNA can now be added before the cutoff point for packaging is reached

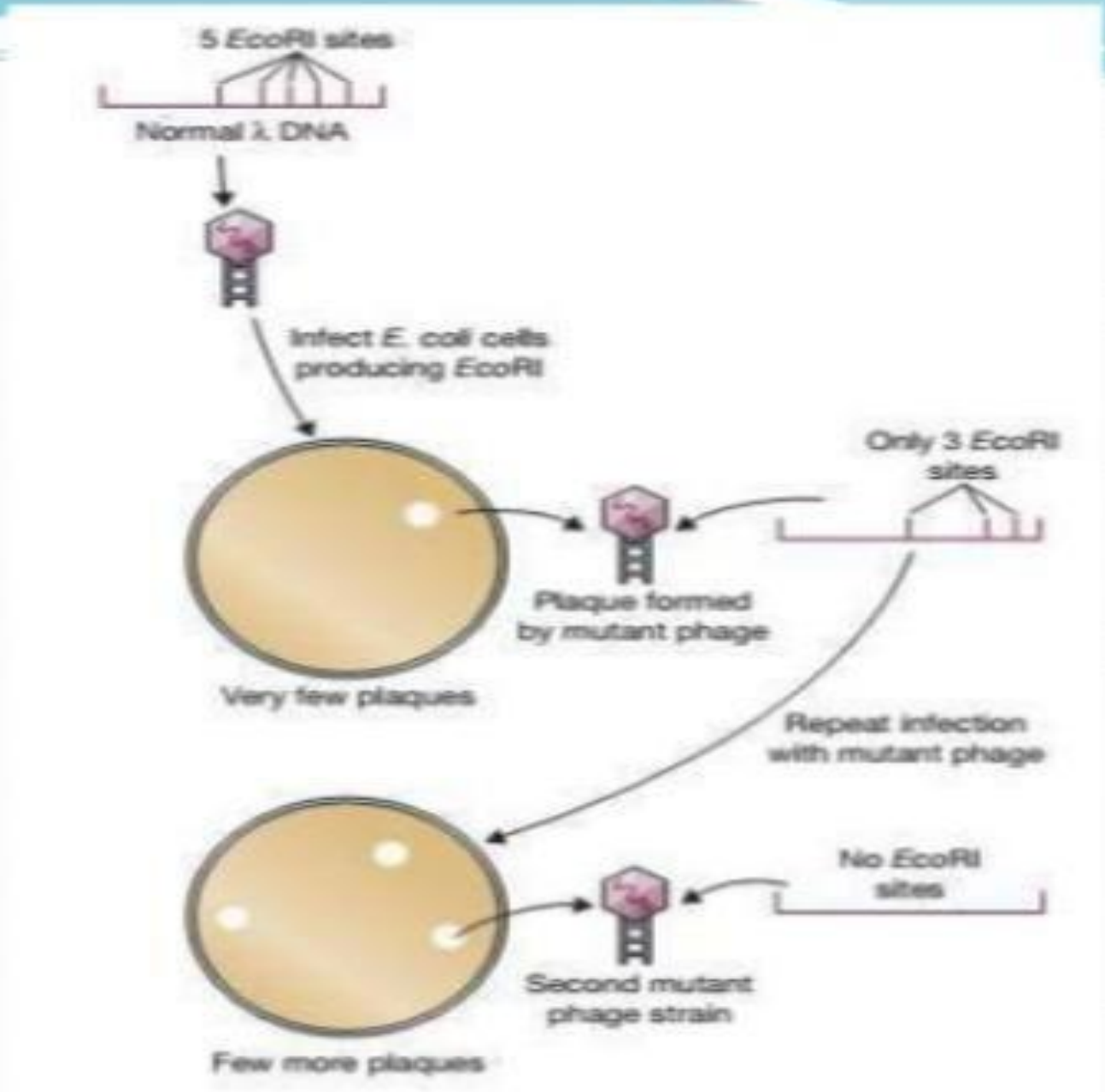

Capsid components
b2
Integration and excision
Early regulation
DNA synthesis
Late regulation
Host lysis

- This “non-essential” region in fact contains most of the genes involved in integration and excision of the prophage from the E. coli chromosome.
- A deleted lambda genome is therefore non-lysogenic and can follow only the lytic infection cycle.

Natural selection can be used to isolate modifie lambda that lack certain restriction sites

- Even a deleted lambda genome, with the non-essential region removed, has multiple recognition sites for most restriction endonucleases.
- If just one or two sites need to be removed, then the technique of in vitro mutagenesis can be used.
- For example, an EcoRI site, GAATTC, could be changed to GGATTC, which is not recognized by the enzyme.
- • However, in vitro mutagenesis was in its early stage. when the first lambda vectors were under development, and even today would not be an efficient means of changing more than a few sites in a single molecule

- Instead, natural selection was used to provide strains of lambda that lack the unwanted restriction sites.
- Natural selection can be brought into play by using as a host an E. coli strain that produces EcoRI.
- Most lambda DNA molecules that invade the cell are destroyed by this restriction endonuclease, but a few survive and produce plaques.
- These are mutant phages, from which one or more EcoRI sites have been lost spontaneously.
- Several cycles of infection will eventually result in lambda molecules that lack all or most of the EcoRI sites.


5 EcoRI sites
Normal λ DNA
Infect E. coli cells producing EcoRI
Only 3 EcoRI sites
Plaque formed by mutant phage
Very few plaques
Repeat infection with mutant phage
No EcoRI sites
Second mutant phage strain
Few more plaques

THANK YOU !

Yeast cloning vectors
Lecture - 5

Why is yeast system required?

- A single cell eukaryote
- Has short life cycle
- Genetically well characterized
- Physiologically well characterized
- Haploid genome has low complexity with size of nearly 12 Mbp
- Easy to grow
- Many auxotrophic and other markers are known
- Several strains harbor 2 µm plasmid
- A source of several strong promoters
- Introduction of naked DNA is easy
- Many yeast genes are functionally expressed in *E.coli*

- Capable of carrying out many post transcriptional and post translational modifications
- Normally secretes very few proteins
- Has relatively high rate of recombination

Transformation of yeast

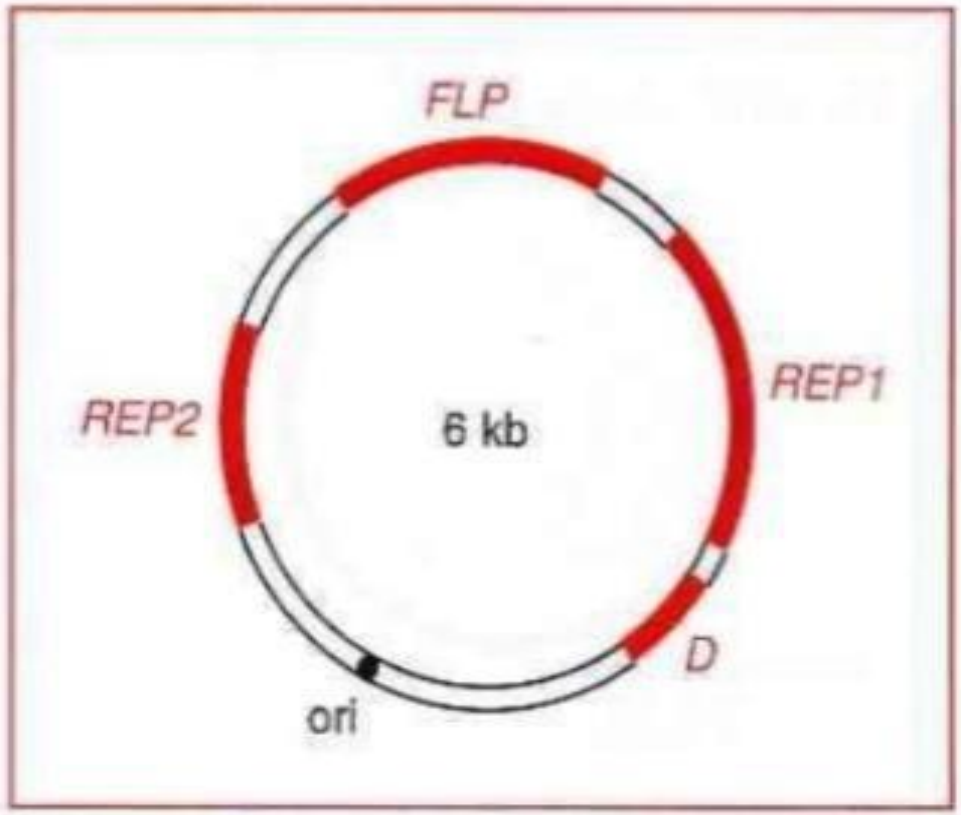
1.Spheroplasts technique

2.Lithium acetate treatment

3.Electroporation

Yeast 2 µm Plasmid

- Covalently closed circular DNA molecule
- Consists of 6318bp of double stranded DNA
- 50-100 copies per haploid cell
- Located in nucleus of yeast cell



Chara YEAST INTEGRATING PLASMIDS (YIps)

•Prese of rep

•Prese sites f

•Prese *coli* ar

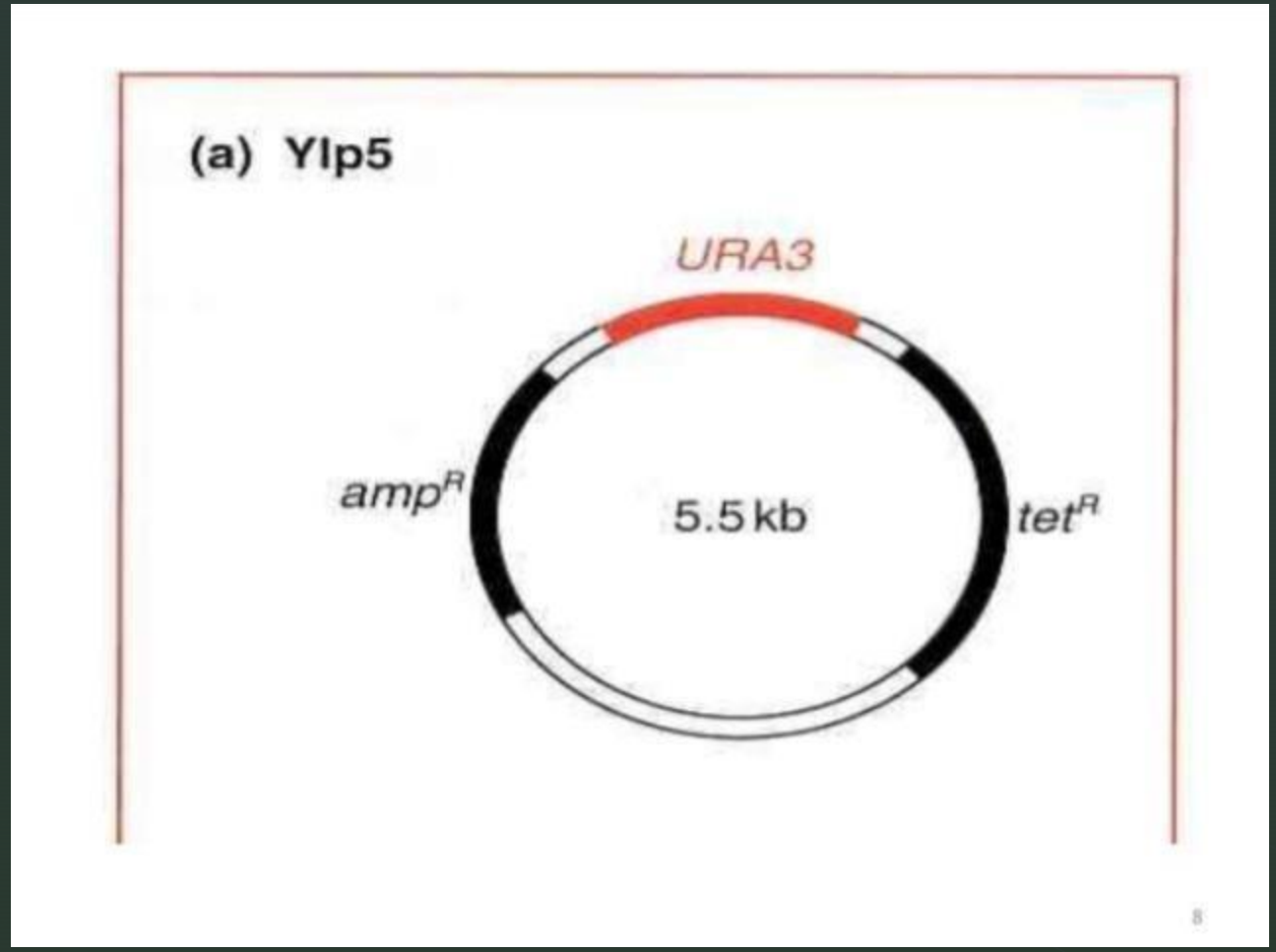
•Have all features of yeast cloning vectors except origin of replication

•These plasmids therefore replicate only by integration into yeast chromosome

•E.g.: YIp5

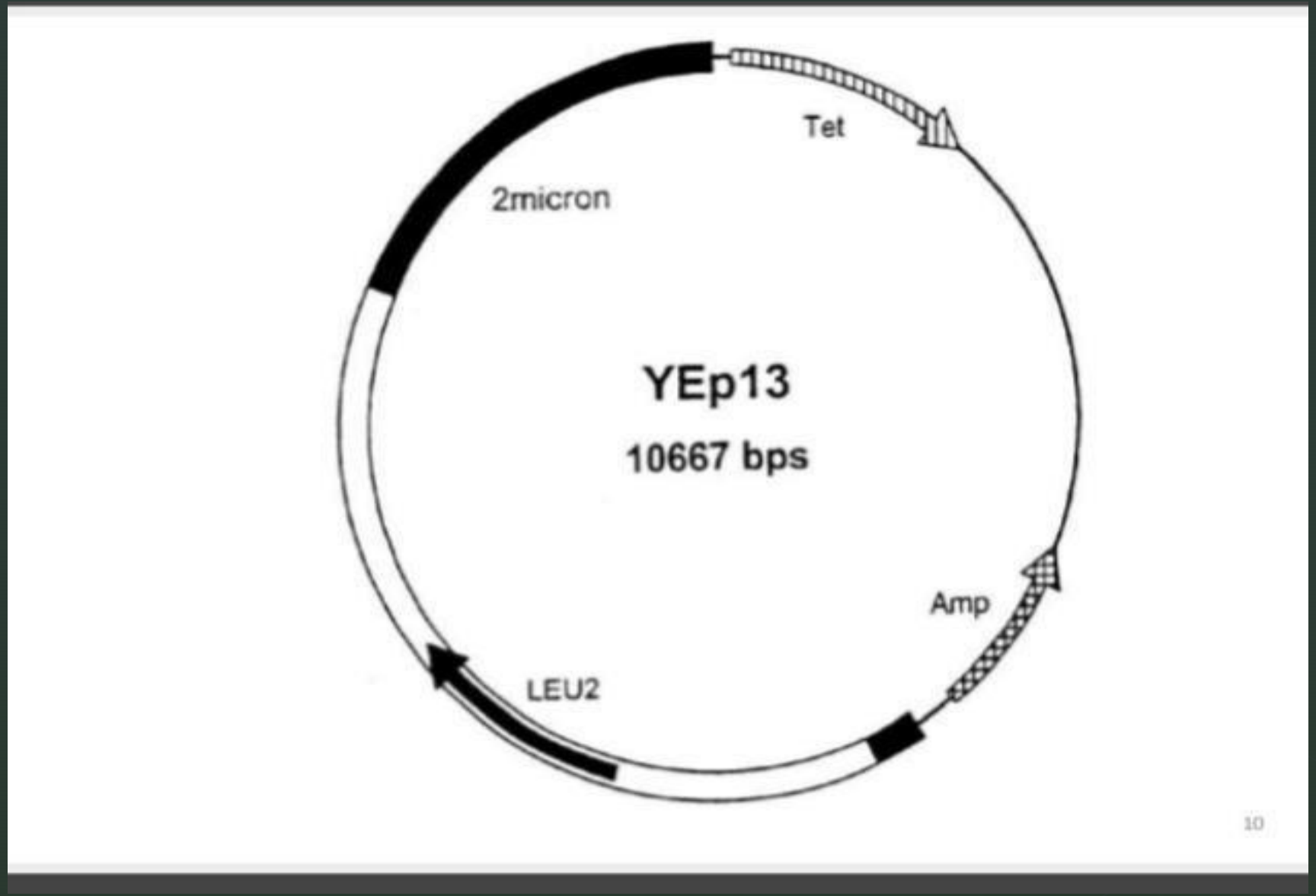
ns

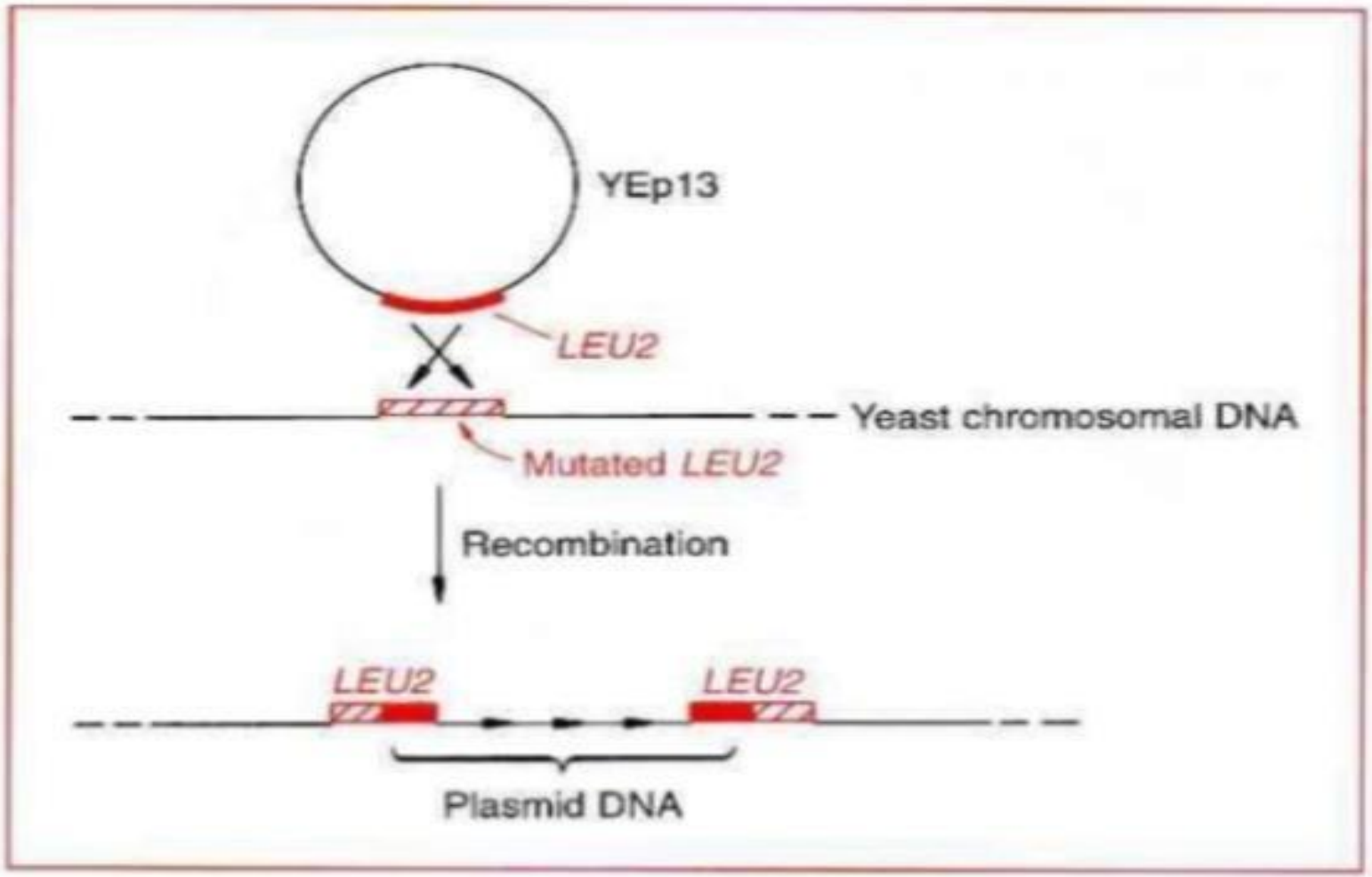
n *E.*


(a) YIp5
URA3
amp^R
5.5 kb
tet^R

YEAST EPISOMAL PLASMIDS (YEps)

- YEps replicate independently but integration into one of the yeast chromosomes can occur
- YEps are designed as double shuttle vectors
- E.g.: YEp13


Tet
2micron
YEp13
10667 bps
Amp
LEU2
10


YEp13
LEU2
Yeast chromosomal DNA
Mutated LEU2
Recombination
LEU2
LEU2
Plasmid DNA

YEAST REPLICATING PLASMIDS (YRps)

- YRps are ARS (Autonomously Replicating Sequence) - based vectors

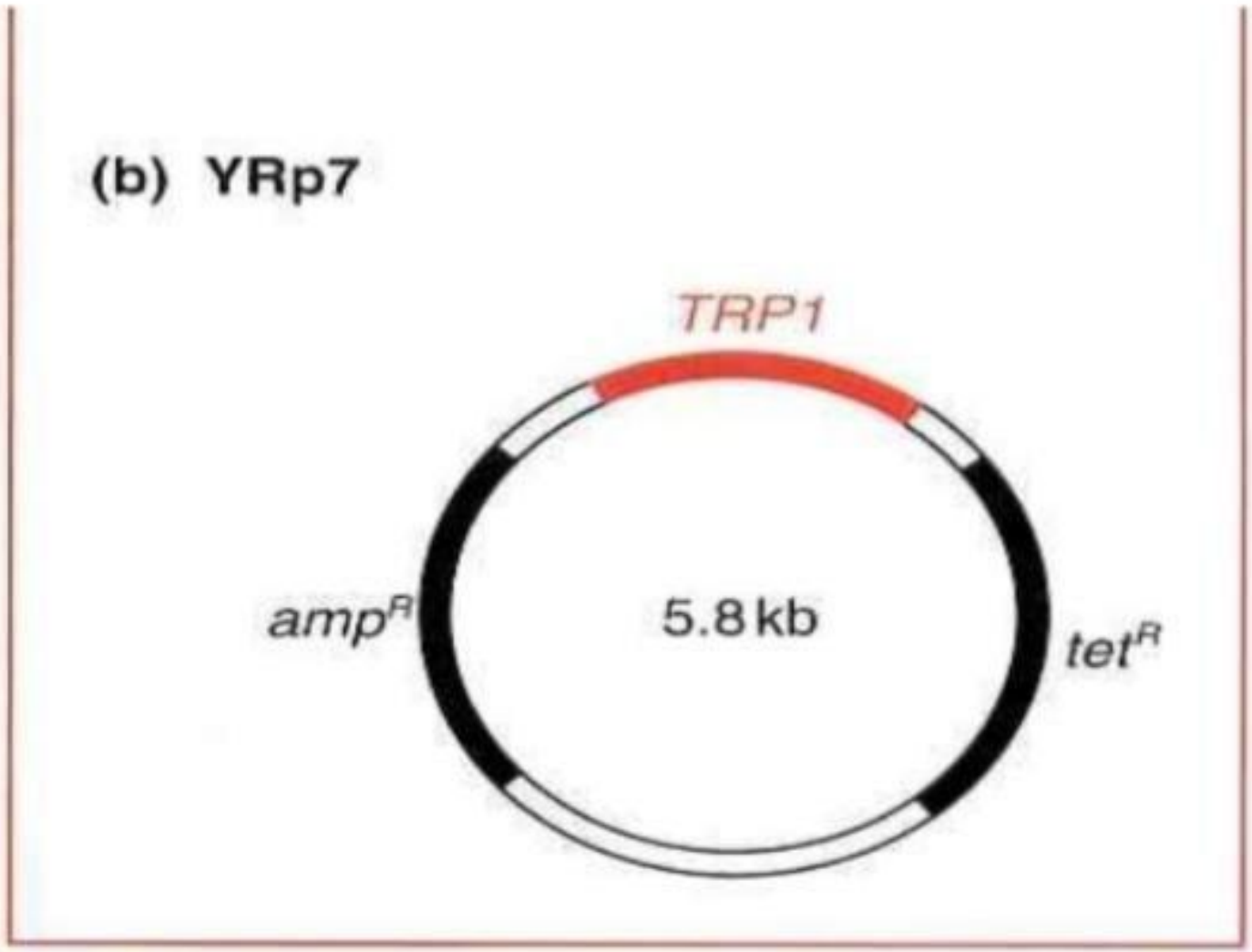
- Exist as low copy number

- Have high transformation efficiency

- Have natural extra chromosomal replication due to ARS

- YRps tend to remain associated with mother yeast cells and have less chances of distribution to daughter cells

- E.g. : YRp7


(b) YRp7
TRP1
amp^R
5.8 kb
tet^R


THANK
YOU

A Presentation on

Expression Vector

Presented By –
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EXPRESSION VECTOR

- A vector used for **expression of a cloned DNA fragment** in a host cell is called as an expression vector.

- These vectors are **frequently engineered** to contain regulatory sequences that act as **promoter and/or enhancer regions** and lead to efficient transcription of the insert gene.

- Expression vectors are used for molecular biology techniques such as **site-directed mutagenesis**.

- The goal of a well-designed expression vector is the production of large amounts of **stable messenger RNA**, and in **extension, proteins**.

- Expression vectors are **basic tools for biotechnology** and the production of proteins.

CHOICE OF EXPRESSION VECTORS

- Strong promoter
- Intact ORF
- Ribosomal binding site
- Termination sequence

EXPRESSION VECTOR COMPONENTS

Goal	Component
Insert cargo into the plasmid and verify the insert sequence accuracy	MCS – restriction sites OR recombination regions 5' and 3' Primer sites for sequence verification
Insert plasmid into cells, enable the plasmid to replicate inside the host, & select for cells carrying the plasmid	•Backbone compatible with cloning method • Origin of replication • Selection marker and/or screening marker
Transcribe mRNA from the plasmid	•Promoter (constitutive or inducible) operator, terminator
Translate mRNA into protein	Ribosome Binding Site, start codon, stop codon
Promote proper folding of nascent protein	•co-expression of chaperones •Solubilization tags •custom-designed synthetic RBS •Codon-optimized ORF
Detect or Purify target protein	•Epitope tags (His) •reporters (GFP)

EXPRESSION VECTOR TWO TYPE

1. PROKARYOTIC EXPRESSION VECTOR

- Bacterial expression system (e.g. E.coli)

2. EUKARYOTIC EXPRESSION VECTOR

- Yeast expression system (e.g. S.cervesiae)
- Viral expression system (e.g. Baculovirus)
- Mammalian cell expression system

Saccharomyces cerevisiae

- It is **the most common eukaryotic system** and there is a great deal of study about this organism

- It is a **single-celled** and behaves like a bacterial culture and can be grown in **relatively simple media** in both **small and large-scale production**

- Well characterized with **many strong regulatory promoters** with **naturally occurring plasmids**

- Carry out **post-translational modifications**

- Secretes very **few of its own proteins**

- **Recognized as safe** by USDA and FDA

Saccharomyces cerevisiae

➢There are **three main classes** of *S. cerevisiae expression* vectors.

- Yeast episomal plasmids (YEps)
- Yeast integrating plasmids (YIps)
- Yeast artificial chromosomes (YACs)

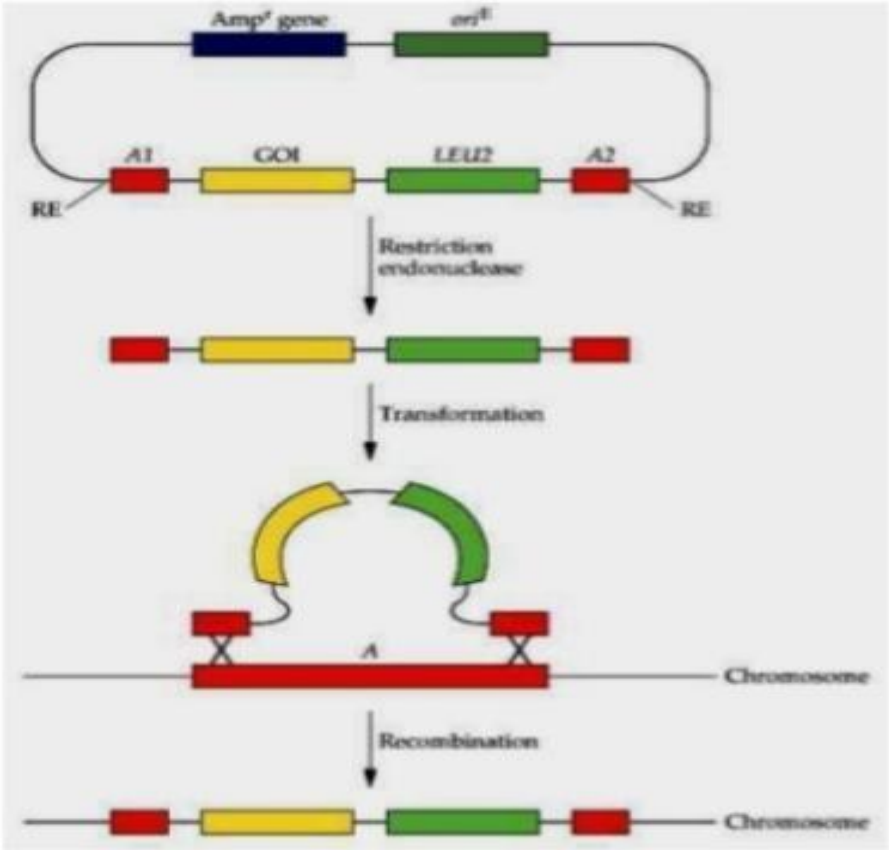
➢ **Yeast episomal plasmid**s have been used extensively for the production of either **intra- or extracellular heterologous proteins**

➢Typically, vectors **function in both** *E. coli and S.cerevisiae*.

Saccharomyces cerevisiae

- The YEps vectors are based on the **high-copy-number 2µm plasmids**
- The vectors replicate independently via a **single origin of replication**.
- There are more than **30 copies per cell**.
- **Selection scheme rely on mutant host strains** that require a particular amino acid (histidine, tryptophan, or leucine) or nucleotide (uracil).
- When a Yep with a wild-type ***LEU2 gene*** *is* transformed into a mutant *leu2 host cell, only cells* that carry plasmid will grow.
- A **Yip vector** is used to integrate a heterologous gene into the host genome to provide a **more reliable production system**.

Integration of DNA with a Yip vector



YAC CLONING SYSTEM

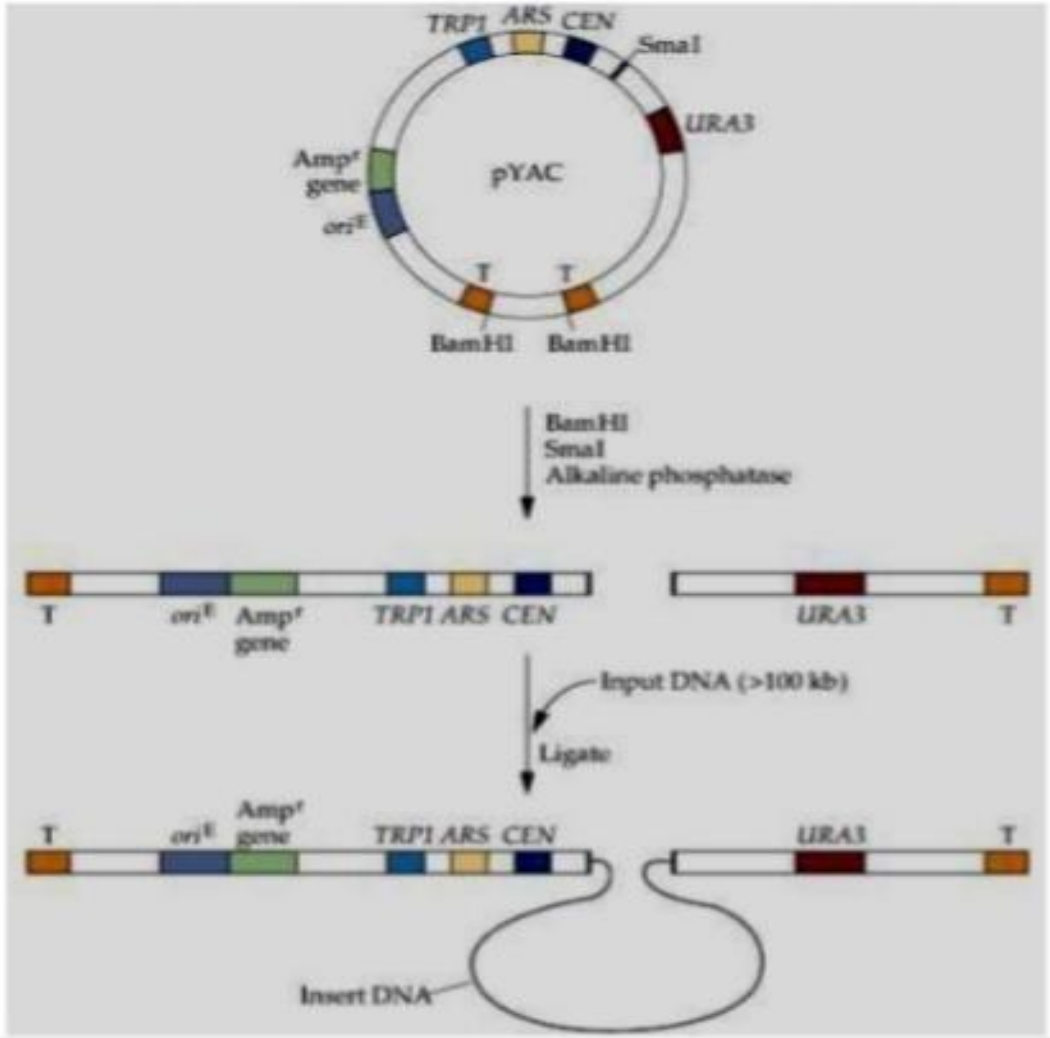
- A YAC is designed to clone a **large segment of DNA (100kb)**, which is then maintained as a separate chromosome in the host yeast cell.

- It is **highly stable** and has been used for the physical mapping of **human genomic DNA**, the analysis of transcription units, and **genomic libraries**.

- It has a sequences that act as **ARS for replication**, **centromere** for cell division, and **telomere** for stability.

- To date, they have **not been used** as expression systems for the **commercial production**.

YAC CLONING SYSTEM



Pichia pastoris Expression Systems

- Though *S. cerevisae* is **successfully used** to produce recombinant proteins for human, it has major **drawbacks**.
- The level of **protein production is low**.
- There is the tendency for **hyperglycosylation** resulting in change of protein function.
- **Proteins** are often **retained in periplasm**, increasing time and **cost for purification**.
- It **produces ethanol** at high cell densities, which is toxic to cells.

Pichia pastoris Expression Systems

- *P. pastoris* is a **methylotrophic yeast** that is able to utilize methanol as a source of carbon and energy.

- **Glycosylation** occurs to a **lesser extent** and the linkages between sugar residues are of the **α-1,2 type.**

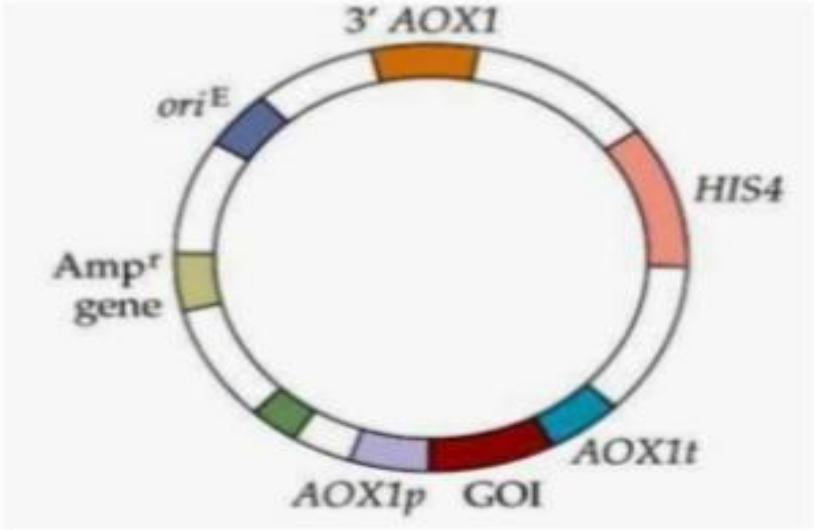
- *P. pastoris* strain was extensively engineered with the aim of developing a "**humanized**" strain that glycosylate proteins in a manner identical to that of human cells.

- It does **not produce ethanol**.

- It normally secretes very few proteins, thus **simplifying the purification** of secreted recombinant proteins.

Pichia pastoris Expression Systems

➢A **double recombination** event between the **AOX1p and AOX1 regions** of the vector and the **homologous segments** of chromosome DNA results in the **insertion of the DNA** carrying the gene of interest and the HIS4 gene.




THANK
YOU

SELECTION OF RECOMBINANT CLONES

INTRODUCTION:

After the introduction of recombinant DNA into the host cells, it is essential to identify those cells which received rDNA molecule - screening or selection.

The vector or foreign DNA present in the recombinant cells expresses certain characters or traits, while non-recombinants do not express the traits.

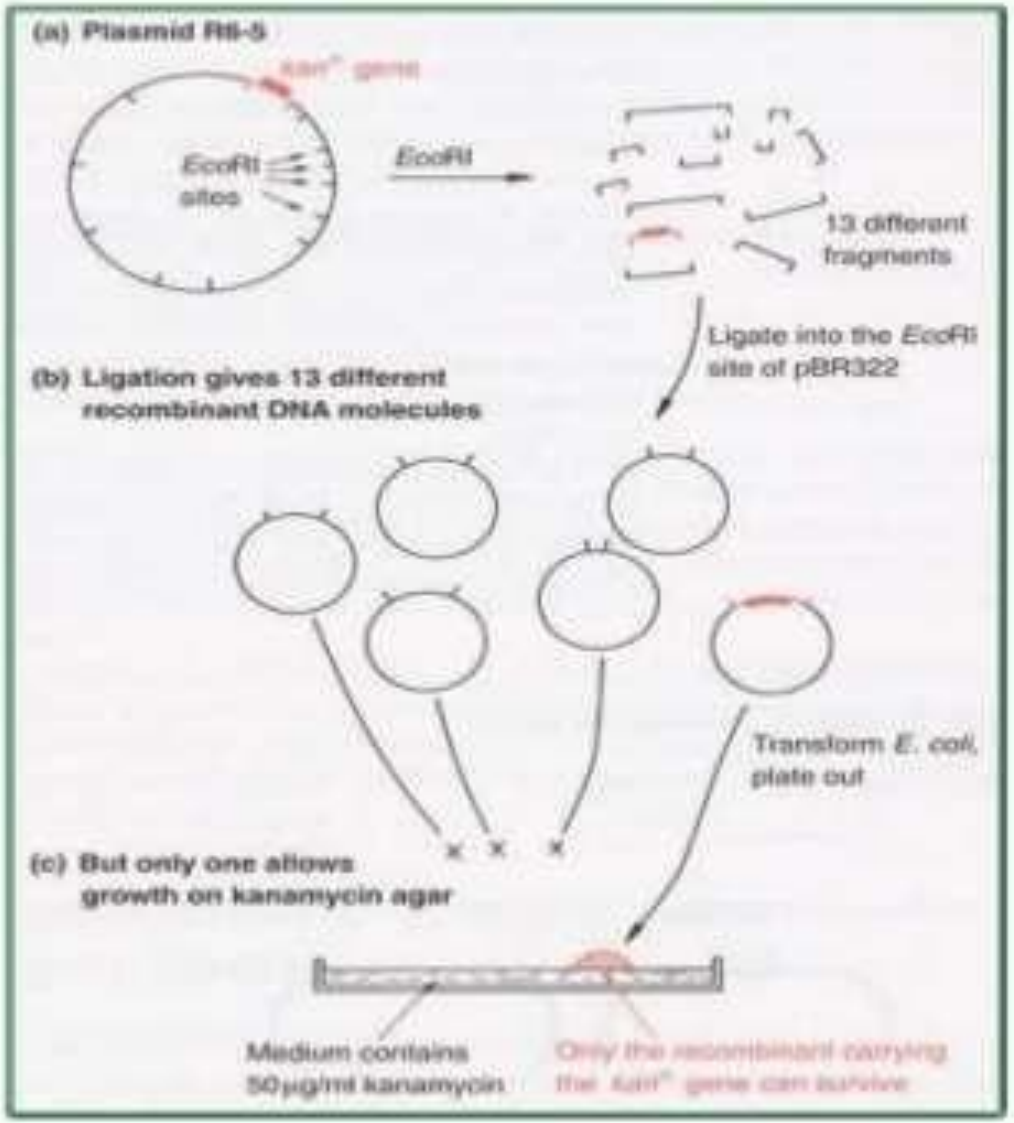
SELECTION METHODS:

Following are the methods for screening of recombinant clones.

- Direct selection of recombinants
- Insertional inactivation selection method
- Blue-white screening
- Colony hybridization technique
- Immunological tests.

DIRECT SELECTION OF RECOMBINANTS:

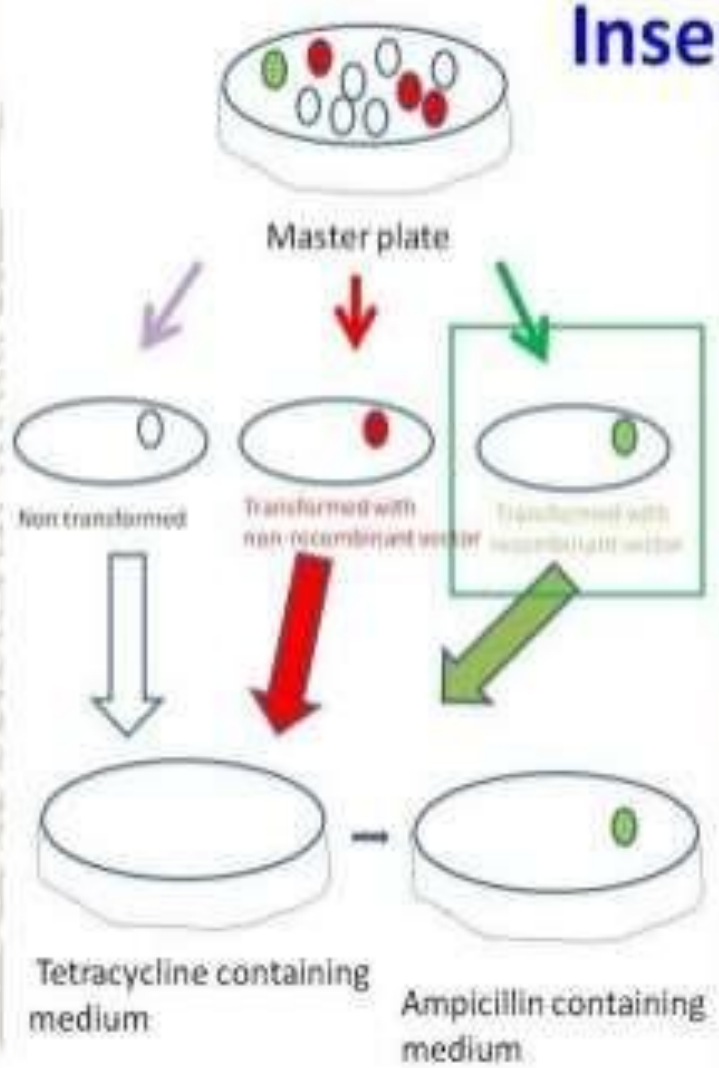
- A circular plasmid containing antibiotic resistance gene can be replicate into the host cell plated on a antibiotic resistance media.
- After transformation cells are plated on medium containing antibiotic.
- Ultimately the transformed cells will grow, i.e, recombinants showing antibiotic resistance.
- Eg: Amp R gene – confers resistance to ampicillin antibiotic in the medium allowing transformed cells or colonies to grow.


(a) Plasmid R6-5
EcoRI sites
EcoRI
13 different fragments
Ligate into the EcoRI site of pBR322
(b) Ligation gives 13 different recombinant DNA molecules
Transform E. coli, plate out
(c) But only one allows growth on kanamycin agar
Medium contains 50 μg/ml kanamycin

INSERTIONAL INACTIVATION:

- Insertional inactivation is the inactivation of a gene upon insertion of another gene in its place or within its coding sequence.
- This helps in selection of recombinant colonies in rRNA technology.
- This is based on expression and non-expression of certain traits or characters. One of the genetic characteristics is disturbed by the introduction of foreign DNA.

Insertional Inactivation



Selection of recombinants

1) Non-transformed:
Cannot grow on ampicillin or tetracycline medium

2) Transformed:
Only transformed colonies can grow in ampicillin or tetracycline containing medium.

a) Transformed with non recombinant or unaltered vector, can grow in both ampicillin and tetracycline containing medium

b)Transformed with recombinant vector carrying our gene of interest. Transformed recombinants can grow only in ampicillin medium and cannot grow on tetracycline medium due to insertional inactivation. So recombinant colonies can be easily selected from the master plate.

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BLUE - WHITE SCREENING

- It is a powerful method for screening recombinants.
- In this method a reporter gene lacZ is inserted in the vector (encodes $\beta galactosidase$).
- $\beta galactosidase$ breaks a synthetic substrate, X gal into an insoluble blue coloured product.
- If a foreign gene is inserted into lacZ, this gene will be inactivated; therefore no blue colour will develop.
- The host cells containing recombinant will form white coloured substrate on the medium containing X-gal.

BLUE WHITE SCREENING:

- The host cells containing non recombinants will turn blue in colour.
- On the basis of colony the recombinants can be selected.

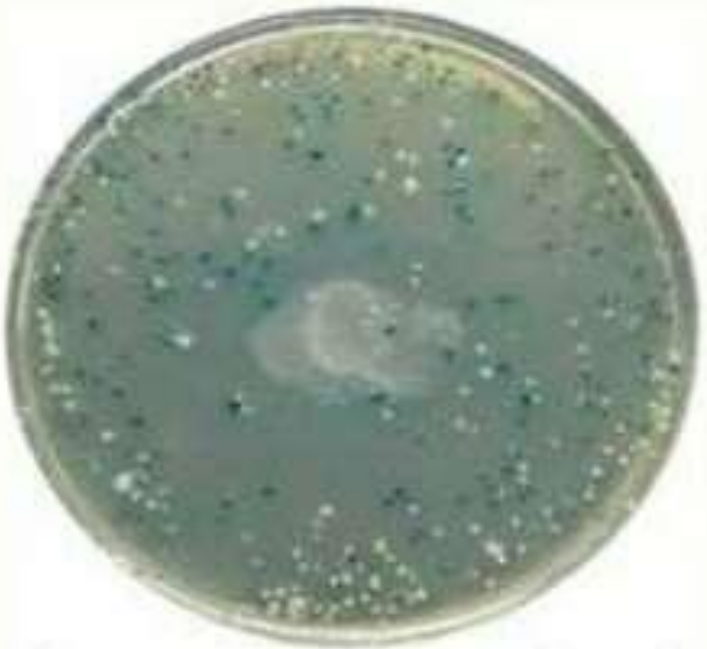

Image source: Wikimedia Commons; Accessed 6/3/15. Author: Stefan Walkowski

COLONY HYBRIDIZATION TECHNIQUE:

- Colony blot hybridization is applied to DNA or RNA released from blotted microbial colonies.
- The microbial colonies are transferred(blotted) to a membrane.
- The cells are lysed in place to release the nucleic acids.
- The RNA or DNA (after denaturation) is fixed to the filter and hybridized with a labelled probe.
- Blocking reagent may be added prior to the probe to prevent unspecific binding.

COLONY HYBRIDIZATION TECHNIQUE:

- Excess probe is washed away and the membrane is visualized by UV or autoradiography.
- Colony blot hybridization can be used for screening clones or bacterial isolates.

IMMUNOLOGICAL TESTS:

- Instead of radio-labelling of DNA molecules, antibodies are used to identify the colonies developed that synthesize antigens encoded by the foreign DNA present in plasmids of the bacterial clones.
    1. Replica plating
    2. Lysis of cells using chloroform vapour/high temperature.
    3. Making gentle contack with a solid support (cellulose filter paper).
    4. Detection of antigen antibody complex by incubating the cellulose filter paper with a radio labelled second antibody.

IMMUNOLOGICAL TESTS:

5. The antibodies which do not react are washed off.

6. The determination of antigen antibody complex is determined by passing through X-ray.

Thank you